

UNIVERSIDADE FEDERAL DO PARANÁ

ANA CAROLINA DOS SANTOS LOURENÇO

EFEITOS DO PLASTIFICANTE DIBUTIL FTALATO (DBP) EM MODELOS IN VIVO
E IN VITRO DE HIPERSENSIBILIDADE DE CONTATO

CURITIBA

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Tese apresentada como requisito parcial à obtenção do grau de Doutor em Farmacologia, Curso de Pós-Graduação em Farmacologia, Setor de Ciências Biológicas da Universidade Federal do Paraná.

Orientador: Prof. Dr. Anderson Joel Martino Andrade

Co-orientador: Prof. Dr. Michel Fleith Otuki

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ATA DE SESSÃO PÚBLICA DE DEFESA DE TESE PARA A OBTENÇÃO DO GRAU DE DOUTOR EM FARMACOLOGIA

No dia vinte e nove de Outubro de dois mil e quinze às 08:30 horas, na sala 107, Centro Politécnico, do Setor de CIÊNCIAS BIOLÓGICAS da Universidade Federal do Paraná, foram instalados os trabalhos de arguição da doutoranda **ANA CAROLINA DOS SANTOS LOURENCO** para a Defesa Pública de sua Tese intitulada: "**Efeitos do plastificante dibutil ftalato (DBP) em modelos in vivo e in vitro de hipersensibilidade de contato**". A Banca Examinadora, designada pelo Colegiado do Programa de Pós-Graduação em FARMACOLOGIA da Universidade Federal do Paraná, foi constituída pelos seguintes Professores Doutores: ANDERSON JOEL MARTINO ANDRADE, DANIELA DE ALMEIDA CABRINI, EUNICE ANDRÉ, JULIANE CENTENO MULLER, DANIELA MORAIS LEME. Dando início à sessão, a presidência passou a palavra a aluna, para que a mesma expusesse seu trabalho aos presentes. Em seguida, a presidência passou a palavra a cada um dos Examinadores, para suas respectivas arguições. A aluna respondeu a cada um dos arguidores. A presidência retomou a palavra para suas considerações finais e, depois, solicitou que os presentes e a doutoranda deixassem a sala. A Banca Examinadora, então, reuniu-se sigilosamente e, após a discussão de suas avaliações, decidiu-se pela **APROVAÇÃO** da aluna. A doutoranda foi convidada a ingressar novamente na sala, bem como os demais assistentes, após o que a presidência fez a leitura do Parecer da Banca Examinadora, outorgando-lhe o Grau de **Doutor em FARMACOLOGIA**. Nada mais havendo a tratar a presidência deu por encerrada a sessão, da qual eu, PATRÍCIA POTT, lavrei a presente ata, que vai assinada por mim e pelos membros da Comissão Examinadora.

Curitiba, 29 de Outubro de 2015.

PATRÍCIA POTT

Secretaria do Programa de Pós Graduação em FARMACOLOGIA

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Prof DANIELA DE ALMEIDA CABRINI

Prof EUNICE ANDRÉ

Prof JULIANE CENTENO MULLER

Prof DANIELA MORAIS LEME

Dedico esse trabalho ao meu maior fã e meu maior ídolo, meu avô Virgílio, que apesar de não estar presente em corpo para ver a minha conclusão do Doutorado, sei que de alguma forma e de algum lugar sempre me acompanha e me protege...

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*“O conhecimento torna a alma jovem e diminui a amargura da velhice.
Colhe, pois, a sabedoria. Armazena suavidade para amanhã.”*

Leonardo da Vinci

RESUMO

A prevalência de doenças alérgicas tais como dermatite, asma e rinite, tem aumentado, e isso poderia ser devido à presença de substâncias químicas ambientais com potencial adjuvante, ou seja, capazes de exacerbar a resposta imune. Ésteres de ftalatos, plastificantes amplamente encontrados no ambiente, têm sido reportados como participantes no desenvolvimento de doenças alérgicas agindo como adjuvantes. A hipersensibilidade de contato (HSC) é uma reação alérgica que resulta de contatos repetidos da pele com substâncias químicas chamadas haptenos, e consiste em duas fases: sensibilização e elicitação. O plastificante dibutil ftalato (DBP) é capaz de aumentar a resposta inflamatória em HSC induzida por alérgeno quando associado em ambas as fases da HSC, mas em doses muito mais altas que aquelas potencialmente relevantes para a exposição humana. Além disso, os mecanismos moleculares envolvidos nesse efeito adjuvante não estão completamente elucidados. O presente estudo pretendeu investigar os efeitos do DBP na fase de elicitação da HSC induzida por oxazolona em modelo animal de edema de orelha, usando camundongos BALB/c e doses mais baixas de DBP. Ainda, investigamos alguns aspectos relacionados aos mecanismos envolvidos nesse efeito adjuvante. Para isso, avaliamos os efeitos do DBP isoladamente ou associado a alérgenos em dois ensaios *in vitro*, o ensaio de ativação de NCTC 2544 (representativo de ativação de queratinócitos) e o ensaio de ativação de THP-1 (substituto de ativação de células dendríticas). Foi também investigado se a indução de estresse oxidativo poderia ser parte do mecanismo molecular responsável pelo efeito adjuvante induzido pelo DBP. Ainda avaliamos a possível participação da ativação dos receptores de potencial transitório A1 e V1 (TRPA1 e TRPV1) no efeito adjuvante do DBP no modelo de HSC induzida por oxazolona. *In vivo*, os parâmetros avaliados foram edema de orelha, 24, 48 e 72 horas após a elicitação, atividade das enzimas N-acetil- β -d-glucosaminidase (NAG) e mieloperoxidase (MPO) (marcadores de infiltração de leucócitos), análise histológica (contagem de células infiltradas, espessura da derme e epiderme) e dosagem de hidroperóxidos de lipídeos (LOOH) e glutathiona reduzida (GSH). Nos experimentos *in vitro*, a produção de interleucina 18 (IL-18) foi avaliada em células NCTC 2544, e a expressão do cluster de diferenciação 86 (CD86) e mRNA de interleucina 8 (IL-8) além da produção de IL-8 e de espécies reativas de oxigênio (EROs) foram avaliados em células THP-1. *In vivo*, o DBP, nas duas maiores doses (0,4 e 4 mg/orelha), foi capaz de aumentar a resposta inflamatória envolvida na HSC induzida por oxazolona, como observado por aumento no edema de orelha, hiperplasia dérmica e epidérmica, contagem de células infiltradas e atividade de NAG e MPO. Antagonistas TRPA1 e TRPV1 reverteram parcial ou completamente, respectivamente, o efeito adjuvante do DBP neste modelo animal de HSC, sugerindo que a ativação destes TRPs seja parte do mecanismo molecular envolvido no efeito adjuvante. *In vitro*, DBP potencializou a ativação de células THP-1, como foi demonstrado pelo aumento da expressão de CD86 e IL-8 mRNA e liberação de IL-8 após exposição a associações de DBP com Citral e imidazolidinil ureia, além do aumento da expressão de CD86 em células THP-1 tratadas com DBP associado a oxazolona. Por outro lado, não foi observado efeito adjuvante nas células NCTC 2544, já que não houve aumento na produção de IL-18 após exposição a p-Fenilenodiamina associado ao DBP, sugerindo que o efeito adjuvante do DBP esteja relacionado com maior ativação de células dendríticas e não de queratinócitos. Nossos resultados indicam que o DBP pode agir

como adjuvante imunológico tanto *in vivo* quanto *in vitro*, e que esse efeito poderia estar relacionado com uma maior ativação de células dendríticas e que envolva ao menos parcialmente a ativação de TRPA1 e TRPV1.

Palavras-chave: dibutil ftalato, hipersensibilidade de contato, efeito adjuvante, queratinócitos, células dendríticas, TRPA1, TRPV1, espécies reativas de oxigênio, estresse oxidativo.

ABSTRACT

The prevalence of allergic diseases, such as atopic dermatitis, asthma and rhinitis has been increasing, and this could be related to the presence of environmental chemicals with immune adjuvancy potential, which could exacerbate the immune response. Phthalate esters, plasticizers widely found in the environment, have been reported to participate in the development of allergic diseases acting as adjuvants. Contact hypersensitivity (CHS) is an allergic reaction resulting from repeated contact of the skin with chemicals called haptens and consisting in two phases: sensitization and elicitation. The plasticizer dibutyl phthalate (DBP) has been shown to enhance the inflammatory response in allergen-induced CHS when associated both in sensitization and elicitation phases, but in much higher doses than those potentially relevant for human exposure. Besides, the molecular mechanisms involved in this adjuvant effect are not fully understood. The present study aimed to investigate the effects of DBP in the elicitation phase of oxazolone induced CHS in an animal model of ear edema, using BALB/c mice and lower doses of DBP. Additionally, we intended to investigate some aspects related to the mechanisms involved in this adjuvant effect. For that, we evaluated the effects of DBP alone or in association with allergens in two *in vitro* assays, NCTC 2544 activation assay (representative of keratinocyte activation) and THP-1 activation assay (surrogate for dendritic cell activation). It was also investigated if the induction of oxidative stress could be part of the molecular mechanisms underlying the adjuvant effect induced by DBP. Moreover, we evaluated the possible participation of transient receptor potential cation channel (TRP) A1 (TRPA1) and V1 (TRPV1) activation by DBP in the animal model of CHS induced by oxazolone. *In vivo*, the parameters evaluated were ear edema 24, 48 and 72 hours after elicitation, activity of the enzymes N-acetyl- β -d-glucosaminidase (NAG) and myeloperoxidase (MPO) (markers of leukocyte infiltration), histological analysis (infiltrated cell count, dermal and epidermal thickness) and lipid hydroperoxide (LOOH) and reduced glutathione (GSH) levels. For *in vitro* experiments, interleukin 18 (IL-18) production was assessed in NCTC 2544 cells and the expression of cluster of differentiation 86 (CD86) and interleukin-8 mRNA besides the release of IL-8 and reactive oxygen species (ROS) production were evaluated in THP-1 cells. *In vivo*, DBP in the two highest doses (0.4 and 4 mg/ear) was able to increase the inflammatory response involved in oxazolone-induced CHS, as observed by increase in ear edema, epidermal and dermal hyperplasia, infiltrated cell count and NAG and MPO activity. TRPA1 and TRPV1 antagonists reversed partially or completely, respectively, the adjuvant effect of DBP in this animal model of CHS, suggesting that the activation of these TRPs are part of the molecular mechanism involved in the adjuvant effect. *In vitro*, DBP potentiated THP-1 cell activation since there was increased expression of CD86 and IL-8 mRNA besides IL-8 release in THP-1 cells exposed to combinations of citral or imidazolidinyl urea with DBP, and increased upregulation of CD86 expression after exposure to DBP associated with oxazolone. On the contrary, no adjuvant effect was seen in the human keratinocyte cell line NCTC 2544, as indicated by the lack of increase in IL-18 production after exposure to p-Phenylenediamine in association with DBP, suggesting that the adjuvant effect of DBP relies on dendritic cells rather than on keratinocytes. In conclusion, our results indicate that DBP can act as an immune adjuvant both *in vivo* and *in vitro*, and this effect could be mainly related to increased dendritic cell activation and at least partially achieved by the activation of TRPA1 and TRPV1.

Key words: dibutyl phthalate, contact hypersensitivity, adjuvant effect, keratinocytes, dendritic cells, TRPA1, TRPV1, reactive oxygen species, oxidative stress.

APRESENTAÇÃO

A presente tese será apresentada na forma de três artigos:

1. The plasticizer dibutyl phthalate (DBP) potentiates chemical allergen-induced THP-1 activation.

2. The plasticizer dibutyl phthalate (DBP) aggravates contact hypersensitivity induced by oxazolone in BALB/c mice.

3. The involvement of transient receptor potential A1 (TRPA1) and V1 (TRPV1) activation in the adjuvant effect of dibutyl phthalate in oxazolone-induced contact hypersensitivity in BALB/c mice.

Para melhor entendimento, é apresentada uma introdução geral e revisão de literatura que se referem a todos os artigos.

Os materiais e metodologia utilizados, resultados, discussão, conclusões e referências encontram-se em cada artigo e representam a íntegra desse trabalho. Para melhor visualização dos resultados relatados nos artigos, após o término de cada um encontra-se incluído o material complementar do mesmo.

Ainda é apresentada uma discussão estendida onde são expostos comentários gerais a respeito dos resultados obtidos nos três artigos, assim como uma conclusão geral da tese.

As referências finais referem-se ao conteúdo apresentado na Introdução, Revisão de Literatura e Discussão da tese, assim como nos materiais complementares.

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1 INTRODUÇÃO

Ésteres de ftalatos são amplamente utilizados como plastificantes e aditivos em uma variedade de produtos, como brinquedos, produtos de limpeza, equipamentos médicos, produtos farmacêuticos, solventes, tintas, repelentes de mosquito e cosméticos. Em razão de sua ubíqua distribuição no ambiente, são considerados agentes com grande potencial para exposição humana (KOO; LEE, 2004; ANDERSON *et al.*, 2001; SILVA *et al.*, 2004; LYCHE *et al.*, 2009; JEONG *et al.*, 2011).

Os ftalatos têm sido utilizados como plastificantes desde a década de 1920. Em 1970, Jaeger e Rubin detectaram a presença de dietilexil ftalato (DEHP) em órgãos e tecidos de dois pacientes hospitalizados que haviam recebido transfusão de sangue. O sangue havia sido estocado em bolsas de policloreto de vinila (PVC) que continham DEHP. Desde a publicação de Jaeger e Rubin (1970), existe grande interesse sobre os possíveis efeitos tóxicos dos ésteres de ftalatos. Hoje se sabe que os ftalatos não se ligam ao PVC e com o tempo e uso, são liberados da matriz plástica, contaminando o ambiente (BAUER; HERRMANN, 1997; BRADBURY, 1996; GIAN *et al.*, 1978; GRIFFITHS; CAMARA; LERNER, 1985).

Alguns ésteres de ftalatos com cadeias curtas, como o dibutil ftalato (DBP), são usados em cosméticos e repelentes de mosquito para uso tópico (INT. J. TOXICOL., 2005; VARTAK; TUNGIKAR; SHARMA, 1994). Além disso, ftalatos com menor peso molecular são mais facilmente absorvidos pela pele (ELSISI; CARTER; SIPES, 1989). Em cosméticos os ftalatos são usados como umectantes e/ou emolientes em cremes hidratantes, como potencializadores de penetração cutânea, como promotores de brilho em esmaltes e como solventes em uma variedade de outros produtos (KOO; LEE, 2004). Shen *et al.* (2007) detectaram a presença de sete tipos de ftalatos em 14 produtos cosméticos incluindo sprays de cabelo, perfumes, desodorantes e cremes hidratantes. Koo e Lee (2004) investigaram diferentes níveis de ftalatos em um grande número de cosméticos, incluindo 42 perfumes disponíveis na Coreia do Sul. De acordo com seus resultados, 11 perfumes (26%) continham níveis detectáveis de DBP. O DBP pode ser encontrado em diversos produtos cosméticos, como perfumes, cremes hidratantes, sprays de cabelo e esmaltes (IPCS, 1997; SATHYANARAYANA *et al.*, 2008).

O DBP, assim como outros ftalatos, tem atraído grande atenção da comunidade científica por seus efeitos como desreguladores endócrinos (FISHER *et al.*, 2003; HEUDORF; MERSCH-SUNDERMANN; ANGERER, 2007; MARTINO-ANDRADE *et al.*, 2009). Um grande número de estudos experimentais avaliaram os efeitos tóxicos do DBP. Este e outros ftalatos são reconhecidamente tóxicos para o desenvolvimento do sistema reprodutor masculino. Ratos machos apresentam alterações e malformações no sistema reprodutor após exposição *in utero* a certos ftalatos (GRAY *et al.*, 2006). Mesmo que muitos aspectos da toxicidade reprodutiva dos ftalatos já tenham sido elucidados, os mecanismos celulares e moleculares responsáveis pelas alterações reprodutivas continuam obscuros.

Além de efeitos reprodutivos e endócrinos, dados experimentais e epidemiológicos indicam que os ftalatos poderiam desempenhar um papel no desenvolvimento e/ou aumento de doenças alérgicas, contribuindo, então, para o aumento na prevalência de doenças alérgicas mediadas por IgE em países industrializados (IMAI *et al.*, 2006; KOIKE *et al.*, 2010; MATSUDA *et al.*, 2010; LI *et al.*, 2014; BORNEHAG *et al.* 2004; JAAKKOLA; KNIGHT, 2004). Além disso, tem sido sugerido que alguns tipos de ftalatos possam agir como adjuvantes, ou seja, que eles possam acelerar, prolongar ou exacerbar uma resposta imune específica (KIMBER; DEARMAN, 2010). Em um estudo com 198 crianças foi reportada uma associação entre sintomas alérgicos e a exposição a certos ftalatos presentes na poeira doméstica. Neste estudo, o DEHP foi relacionado com o aparecimento de asma, e o butil benzil ftalato (BBzP) foi associado a rinite e eczema (BORNEHAG *et al.*, 2004). Foi também demonstrado que alguns ésteres de ftalatos podem aumentar a produção de anticorpos quando injetados juntamente a certos antígenos em camundongos (LARSEN *et al.*, 2001; 2002). Ainda, foi demonstrado que alguns ésteres de ftalatos podem agir como adjuvantes na sensibilização por fenetil isotiocianato (MATSUDA *et al.*, 2010) ou isotiocianato de fluoresceína (FITC) (IMAI *et al.*, 2006) aumentando a intensidade da resposta inflamatória em modelos animais de hipersensibilidade de contato. Yanagisawa *et al.* (2008) também demonstraram que a exposição de camundongos ao DEHP durante a lactação foi capaz de aumentar a intensidade da dermatite de contato na prole exposta, sugerindo que os efeitos tóxicos resultantes da exposição perinatal aos ftalatos possam ir além das alterações no desenvolvimento do sistema reprodutor masculino.

A dermatite de contato alérgica (DCA), também conhecida como hipersensibilidade de contato (HSC), é uma reação inflamatória cutânea resultado de contatos repetidos ou prolongados da pele com substâncias químicas de baixo peso molecular denominadas haptenos (SAINT-MEZARD *et al.*, 2004; LEPOITTEVIN; LEBLOND, 1997) e é considerada uma das mais importantes manifestações de imunotoxicidade em humanos (KIMBER, 2002). A dermatite de contato alérgica é uma hipersensibilidade do tipo tardia, mediada por linfócitos T antígeno específicos (KARLBERG *et al.*, 2008). O início da hipersensibilidade de contato é dado pela aplicação tópica de haptenos sensibilizantes à pele. Duas fases são necessárias para atingir a reação máxima da hipersensibilidade de contato: as fases de sensibilização e elicitação. A sensibilização ocorre após o primeiro contato da pele com o hapteno e leva a ativação e expansão de células T específicas nos linfonodos. A elicitação ocorre algumas horas após um contato subsequente da pele com o mesmo hapteno, que resulta no recrutamento das células T específicas. A elicitação ou indução leva 72 horas em humanos e 24 a 48 horas em camundongos. A reação inflamatória persiste por dias e diminui progressivamente através de mecanismos fisiológicos de regulação negativa.

Diversos tipos celulares estão envolvidos na hipersensibilidade de contato. Queratinócitos tem papel importante em todas as fases da dermatite de contato alérgica. Eles respondem a alérgenos produzindo citocinas pró-inflamatórias, que são importantes para a maturação das células dendríticas e sua migração para os linfonodos, onde há a ativação da resposta imune específica (VANDEBRIEL; VAN OCH; VAN LOVEREN, 2005). Células dendríticas são reconhecidas como apresentadoras de antígenos na resposta imune adaptativa por sua capacidade de estimular linfócitos naïve (BANCHEREAU *et al.*, 2000).

Evidências indicam um papel central do estresse oxidativo na dermatite de contato induzida por alérgenos e doenças inflamatórias cutâneas (OKAYAMA, 2005; BYAMBA *et al.*, 2010; CORSINI *et al.*, 2013b). Muitos estudos *in vitro* têm revelado que a produção de espécies reativas de oxigênio (EROs) é induzida por alérgenos de contato (CORSINI *et al.*, 2013), e vários indícios apoiam a participação de EROs na patogênese da dermatite de contato. O estresse oxidativo poderia ser o gatilho, já que leva à ativação de fatores de transcrição e vias de sinalização, incluindo NF- κ B e p38 MAPK, que promovem a liberação de citocinas e quimiocinas (GLOIRE; LEGRAND-POELS; PIETTE, 2006; KIM *et al.*, 2012). A maioria dos alérgenos de

contato se ligam ao grupo tiol da cisteína (DIVKOVIC *et al.*, 2005), o que pode levar à depleção de glutatona, estresse oxidativo, dano tecidual e aumento de inflamação. Além disso, já foi reportado que substâncias antioxidantes são capazes de prevenir o processo de maturação de células dendríticas durante a apresentação de antígenos e suprimir a resposta imune adaptativa em animais (MATSUE *et al.*, 2007). Também já foi proposto que um dos papéis biológicos da dermatite de contato alérgica seja um mecanismo de defesa contra substâncias químicas capazes de alterar o balanço redox celular (SASAKI; AIBA, 2007).

Além da participação de estresse oxidativo no desenvolvimento da hipersensibilidade de contato, existem evidências de que o sistema imune está conectado ao sistema nervoso periférico (BERESFORD *et al.*, 2004; LIU *et al.*, 2006). Já foi sugerido que neurônios sensoriais que detectam estímulos nocivos poderiam estar envolvidos com o início de algumas alergias (PALM *et al.*, 2012). Foi reportado que a HSC pode ser suprimida com a deleção de fibras sensíveis a capsaicina (BERESFORD *et al.*, 2004). Estas fibras expressam o receptor de potencial transitório (TRP) do tipo vanilóide 1 (TRPV1), um canal de cálcio permeável envolvido na nocicepção causada por estímulos químicos e térmicos, também conhecido como receptor de capsaicina (CAP) (CATERINA *et al.*, 1997; CLAPHAM, 2003). Foi demonstrado que alguns neurônios sensoriais que expressam TRPV1 também podem expressar TRPA1 (receptor de potencial transitório relacionado à proteína anquirina 1), outro membro da família de canais permeáveis a cálcio TRP (STORY *et al.*, 2003), que pode ser ativado por substâncias químicas como alil isotiocianato (AITC) e cinamaldeído (BANDELL *et al.*, 2004; JORDT *et al.*, 2004). A ativação de TRPA1 ou TRPV1 pode resultar na liberação de neuropeptídeos de terminações nervosas periféricas (BAUTISTA *et al.*, 2005, ZYGMUNT *et al.*, 1999), como o peptídeo relacionado ao gene da calcitonina (CGRP) e a substância P (SP), cuja participação na resposta inflamatória a HSC já foi demonstrada (MARUYAMA *et al.*, 2007; MIKAMI *et al.*, 2011; LIU *et al.*, 2013). Ainda, tem sido reportado que a dessensibilização de TRPA1 e TRPV1 através de tratamento local com AITC e CAP, respectivamente, suprimiu a sensibilização a FITC em camundongos BALB/c (MARUYAMA *et al.*, 2007).

Alguns modelos animais de inflamação cutânea auxiliam na identificação de substâncias sensibilizantes, pois promovem condições que se assemelham com alguns tipos de dermatites observadas em humanos (VANE; BOTTING, 1998;

BOUCLIER *et al.*, 1990). Dentre as substâncias sensibilizantes, a oxazolona é frequentemente utilizada por ser capaz de induzir as duas fases da hipersensibilidade de contato. A oxazolona é considerada um hapteno forte, com propriedades pró-inflamatórias potentes (ZHANG; TINKLE, 2000). O modelo de edema de orelha induzido pela oxazolona (FUJII *et al.*, 2002) mimetiza a reação de hipersensibilidade de contato do tipo tardia, e pode ser utilizado para identificar substâncias sensibilizantes baseado na capacidade de aumento do espessamento da orelha de animais sensibilizados (KIMBER *et al.*, 1999), que vem acompanhado de infiltração celular e liberação de diversas citocinas (WEBB *et al.*, 1998; FUJII *et al.*, 2002; BAS *et al.*, 2007). No entanto, seria importante tanto do ponto de vista econômico quanto ético, desvendar marcadores biológicos que sejam detectáveis *in vitro* para discriminar substâncias sensibilizantes as quais os humanos estejam expostos. Corsini *et al.* (2009) propuseram um ensaio *in vitro* baseado na produção de interleucina 18 (IL-18) na linhagem queratinocítica humana NCTC 2544 para identificar sensibilizantes de contato e distingui-los de irritantes e alérgenos respiratórios (GALBIATI *et al.*, 2011; CORSINI *et al.*, 2013a). IL-18 favorece a resposta imune do tipo Th-1 aumentando a secreção de mediadores pró-inflamatórios como TNF- α , IL-8 e IFN- γ (CUMBERBATCH *et al.*, 2001). Mitjans *et al.* (2008) reportaram na linhagem celular monocítica THP-1 (usada como substituta de células dendríticas), uma produção seletiva da quimiocina interleucina 8 (IL-8) por alérgenos de contato em paralelo a um aumento na expressão da proteína de membrana CD86 (cluster de diferenciação 86) e propuseram esse ensaio como um possível teste *in vitro* de sensibilização. IL-8 é um potente atrativo para neutrófilos e diferentes subtipos de células T.

Estudos envolvendo exposição crônica a ésteres de ftalatos mostram que essas substâncias são bem absorvidas pela pele (ELSISI; CARTER; SIPES, 1989; DOAN; BRONAUGH; YOURICK, 2010; BEYDON; PAYAN; GRANDCLAUDE, 2010; PAN *et al.*, 2014). De acordo com dados epidemiológicos, ésteres de ftalatos não tem o potencial de causar irritação dérmica ou sensibilização da pele (MEDEIROS; DEVLIN; KELLER, 1999; GEIER *et al.*, 2004). Além disso, agências de regulamentação já investigaram o possível efeito sensibilizante dos ftalatos, mas nenhuma evidência de influência sobre a resposta inflamatória na pele por essas substâncias foi encontrada (CPSC, 2010). Assim, em alguns países, como no Brasil, essas substâncias ainda são permitidas em diversas formulações cosméticas. No

entanto, esses estudos não incluíram o possível efeito adjuvante dos ftalatos em modelos de hipersensibilidade de contato. Mesmo os ftalatos não sendo capazes de induzir hipersensibilidade, o fato que essas substâncias possam ser capazes de exacerbar a resposta inflamatória em modelos animais de hipersensibilidade de contato (CHOWDHURY; STATHAM, 2002; IMAI *et al.*, 2006; MATSUDA *et al.*, 2010; LI *et al.*, 2014) sugere que a toxicidade dérmica dos ésteres de ftalatos necessite de maior atenção e avaliação.

Com esse estudo, pretendemos investigar o possível efeito adjuvante dos ésteres de ftalatos no desenvolvimento da hipersensibilidade de contato induzida por oxazolona em modelo animal de edema de orelha, avaliando doses mais baixas de DBP (0,4 mg/animal) (KOO; LEE, 2004; INT. J. TOXICOL., 2005). Além disso, investigamos alguns aspectos relacionados ao mecanismo do efeito adjuvante do DBP em processos alérgico-inflamatórios. Para isso, foram avaliados os efeitos do DBP sozinho ou em associação com alérgenos em dois ensaios *in vitro* representativos de respostas queratinocíticas e dendríticas, a linhagem queratinocítica humana NCTC 2544 e a linhagem monocítica humana THP-1, respectivamente. Foi também investigado se a indução de estresse oxidativo poderia ser parte do mecanismo molecular do efeito adjuvante induzido por DBP. Isso porque diversos autores já reportaram relação entre ftalatos e estresse oxidativo demonstrando aumento da geração de espécies reativas de oxigênio (SEO *et al.*, 2004; TETZ *et al.*, 2013). Outros têm associado a presença de diversos metabólitos monoésteres de ftalatos detectados na urina com aumento de marcadores de inflamação e estresse oxidativo (HAUSER *et al.*, 2007; FERGUSON; LOCH-CARUSO; MEEKER, 2011). Também avaliamos a possível participação da ativação dos receptores TRPA1 e TRPV1 no efeito adjuvante do DBP no modelo de edema de orelha induzida por oxazolona, já que já foi demonstrada a capacidade do DBP em ativar tanto TRPA1 quanto TRPV1 *in vitro* (SHIBA *et al.*, 2009).

Os resultados obtidos nesse estudo contribuem para uma maior compreensão sobre o efeito adjuvante do dibutil ftalato em modelos de hipersensibilidade de contato *in vivo* e *in vitro*.

2 REVISÃO DE LITERATURA

2.1 FTALATOS

2.1.1 Estrutura e propriedades físico-químicas

Estruturalmente, os ésteres de ftalatos consistem em grupos ésteres pareados ligados a um anel benzeno (AUTIAN, 1973; KLUWE, 1982). As configurações meta e para são conhecidas como isoftalatos e tereftalatos, respectivamente. No entanto, o termo “éster de ftalato” é restrito a configuração orto da molécula. Os ftalatos são sintetizados comercialmente pela condensação de um álcool apropriado com o anidrido ftálico, como indicado na figura abaixo (FIGURA 01) (AUTIAN, 1973; KLUWE, 1982).

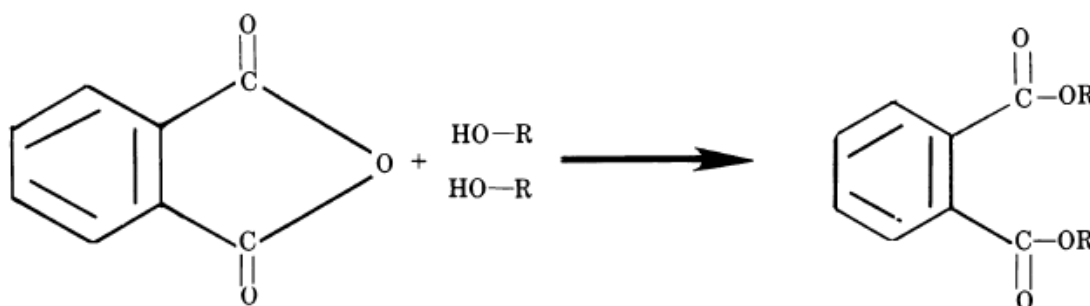


FIGURA 01 – REAÇÃO QUÍMICA DE SÍNTESE DOS ÉSTERES DE FTALATO
 FONTE: Kluwe (1982).

A maioria dos ésteres são incolores, apresentam baixa volatilidade e são praticamente insolúveis em água devido a sua estrutura lipofílica, sendo solúveis, portanto, em solventes orgânicos e óleos (AUTIAN, 1973; LYCHE *et al.*, 2009). A baixa solubilidade em água está relacionada ao comprimento da cadeia lateral, sendo que quanto maior a cadeia e, por consequência, maior peso molecular, menor é a solubilidade do ftalato em água (LYCHE *et al.*, 2009). Como exemplo, o dibutil ftalato (DBP) e o dimetil ftalato (DMP) apresentam certa solubilidade em água

enquanto o di-2-etilhexil ftalato (DEHP) e o di-isononil ftalato (DINP) são insolúveis em água (AUTIAN, 1973; LYCHE *et al.*, 2009).

2.1.2 Tipos, fontes e usos

Os ftalatos são amplamente utilizados como plastificantes, sendo adicionados ao policloreto de vinila (PVC) para conferir maleabilidade e durabilidade ao polímero (SILVA *et al.*, 2004; LYCHE *et al.*, 2009). Podem ser encontrados também como solventes, óleos lubrificantes, estabilizante de cor e fragrância e como detergentes em produtos de cuidados pessoais (como, por exemplo, cosméticos, loções e perfumes) (KOO; LEE, 2004; DUTY *et al.*, 2005). Em cosméticos, os ftalatos atuam como umectantes e/ou emolientes em cremes hidratantes, como potencializadores da penetração cutânea e para promoção de brilho e impedimento de ressecamento e quebra de esmaltes (KOO; LEE, 2004).

Quando são incorporados ao PVC, os ftalatos não se ligam covalentemente ao polímero e por isso são facilmente liberados no meio ambiente, principalmente quando entram em contato com substâncias lipofílicas, resultando na exposição humana e de animais (SHEA; COMMITTEE ON ENVIRONMENTAL HEALTH, 2003; HEUDORF, MERSCH-SUNDERMANN; ANGERER, 2007; LYCHE *et al.*, 2009; JEONG *et al.*, 2011). Além disso, eles são liberados diretamente no ambiente durante a produção e utilização e após o descarte de PVC e outros produtos que os contém (SHEA; COMMITTEE ON ENVIRONMENTAL HEALTH, 2003).

O uso dos vários tipos de ftalatos está relacionado ao seu peso molecular. Os ftalatos de baixo peso molecular, tais como: DMP, DBP e o di-etil ftalato (DEP), tendem a ser usados como solventes em cosméticos e em inseticidas, como plastificantes em acetato de celulose, como promotores de liberação controlada em produtos farmacêuticos, além de também serem encontrados no PVC (ATSDR, 1995; ATSDR, 2001; LYCHE *et al.*, 2009). Já os ftalatos de alto peso molecular, como DEHP, DiNP, butilbenzil ftalato (BBzP) e di-isodecil ftalato (DiDP), são mais utilizados em materiais de construção (como, revestimentos de paredes e pavimentos) e em uma grande gama de produtos a base de PVC, incluindo vestuários (por exemplo: calçados e capas de chuva), embalagens de alimentos,

produtos infantis (como os brinquedos) e equipamentos médicos-hospitalares (como bolsas de sangue) (ATSDR, 1995; ATSDR, 2001; PREUSS; KOCH; ANGERER, 2005; LYCHE *et al.*, 2009).

2.1.3 Efeitos dos ftalatos sobre o organismo humano

Os ésteres de ftalato são utilizados em vários produtos industrializados (ANDERSON *et al.*, 2001; SILVA *et al.*, 2004), sendo a exposição humana a essas substâncias inevitável, podendo ocorrer por ingestão, inalação ou absorção cutânea (ANDERSON *et al.*, 2001; SILVA *et al.*, 2004; LYCHE *et al.*, 2009; JEONG *et al.*, 2011).

Os ftalatos têm atraído grande atenção da comunidade científica não somente devido à ampla possibilidade de exposição humana, bem como pelos possíveis efeitos desreguladores endócrinos que podem ocasionar alterações no desenvolvimento reprodutivo masculino (MYLCHREESTER *et al.*, 1999; FISHER *et al.*, 2003; HEUDORF, MERSCH-SUNDERMANN; ANGERER, 2007; MARTINO-ANDRADE *et al.*, 2009). Desreguladores endócrinos são substâncias capazes de mimetizar ou bloquear a ação de um hormônio endógeno, interferindo com o funcionamento normal do sistema endócrino (ATSDR, 2001). Os ftalatos exibem uma ação anti-androgênica, sendo que estudos em animais demonstraram que a exposição gestacional e lactacional ao DBP, BBzP e DEHP provocam toxicidade testicular, com sintomas característicos da Síndrome de Disgenesia Testicular (TDS) - hipospádia, criptorquidia e função testicular comprometida (FOSTER *et al.*, 2001; FISHER *et al.*, 2003; MARTINO-ANDRADE *et al.*, 2009).

No entanto, estudos epidemiológicos e experimentais recentes também têm demonstrado possíveis associações entre a exposição a alguns ftalatos e doenças inflamatórias como asma e dermatites (BORNEHAG *et al.*, 2004; TAKANO *et al.*, 2006; KOLARIK *et al.*, 2008; YANAGISAWA *et al.*, 2008). Além disso, existem dados indicando um possível aumento na incidência de doenças alérgicas, principalmente entre crianças e jovens, ao longo das últimas décadas (BEASLEY; ELLWOOD; ASHER, 2003). Substâncias químicas como ftalatos poderiam aumentar a potência

de alérgenos e, assim, desempenhar um papel no desenvolvimento e/ou agravamento de doenças alérgicas (CASILLAS *et al.*, 1999).

2.1.4 Dibutil Ftalato (DBP)

O di-n-butil ftalato, mais conhecido como DBP é sintetizado através da reação do n-butanol com anidrido ftálico (FIGURA 02) (NTP, 2003), possui peso molecular de 278,34 g/mol (AUTIAN, 1973; IPCS, 1997; ATSDR, 2001), é solúvel em solventes orgânicos como álcool, éter, acetona e benzeno, e apresenta solubilidade em água a 20°C de 10mg/litro (IPCS, 1997; ATSDR, 2001).

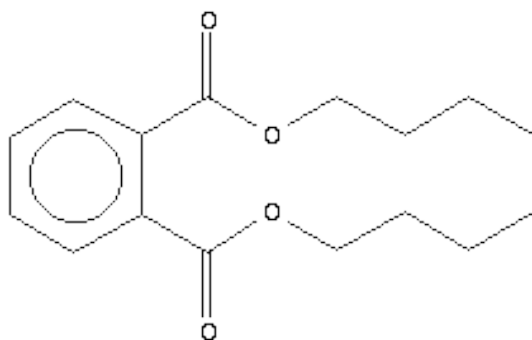


FIGURA 02 – ESTRUTURA QUÍMICA DO DIBUTIL FTALATO
FONTE: NIST (2011)

Uma variedade de produtos de consumo contém DBP, incluindo plástico envoltório de alimentos, colas, tintas, corantes à base de óleo, perfumes, emolientes de pele, sprays de cabelo, esmaltes de unha e repelentes de insetos (IPCS, 1997; ATSDR, 2001).

Em estudo mais recente realizado por Koniecki e colaboradores (2011), foram mensuradas as concentrações de 18 tipos de ftalatos, entre eles o DBP, em 252 produtos cosméticos e de higiene pessoal, incluindo produtos para o cuidado de bebês no Canadá. O DBP estava presente em nove destes produtos, sendo que a maior parte eram em esmaltes de unha com concentração de 24304 µg/g de produto. Também foi encontrado em menores concentrações (36 µg/g) em produtos como spray e mousses de cabelo, produtos para a limpeza de pele e xampus

infantis. Nesse mesmo estudo, foi calculada uma exposição diária de DBP em 0,36 µg/kg de peso corpóreo (KONIECKI *et al.*, 2011).

Em um estudo realizado por Koo e Lee (2004), 26% dos perfumes (11 de 42 perfumes analisados) continham níveis detectáveis de DBP, sendo que a concentração média de DBP encontrada foi de 0,44 mg/mL e a utilização mediana de perfumes pela população, estimada através de questionário aplicado, foi de 1 mL/dia. Portanto, a quantidade de DBP aplicada em indivíduos que utilizam determinados tipos de perfumes pode ser de aproximadamente 0,4 mg/dia. Considerando que alguns indivíduos utilizam quantidades ainda maiores de perfumes e outros cosméticos contendo DBP, esse nível de exposição tópica pode ser ainda maior (KOO; LEE, 2004).

Além disso, em 2005, o International Journal of Toxicology publicou em sua revisão anual sobre a segurança de ingredientes em produtos cosméticos uma estimativa sobre a exposição humana aos ftalatos presentes em cosméticos. Os dados foram fornecidos em quantidades de ftalatos presentes em diferentes tipos de cosméticos (perfumes, desodorantes, spray de cabelo e esmalte), a quantidade efetivamente aplicada sobre a pele e as taxas de absorção dessas substâncias (INT. J. TOXICOL., 2005). De acordo com essas estimativas, a aplicação dérmica de ftalatos para esses produtos varia entre 0,1 e 0,45 mg/dia, o que está de acordo com as estimativas de Koo e Lee (2004).

2.1.5 Farmacocinética do DBP

Quando administrados pela via oral são hidrolisados por lipases e absorvidos quase totalmente na forma de seus metabólitos correspondentes (os monoésteres de ftalatos) (ROWLAND; COTTRELL; PHILLIPS, 1977), sendo que para o DBP, o metabólito mais encontrado no plasma é o MBP (monobutil ftalato) (FENNEL *et al.*, 2004). Extensa absorção oral é observada, indicada pelo fato de que, em ratos, 63-97% de uma dose oral foi quantificada na urina 24h após a administração (FOSTER *et al.*, 1983; TANAKA; MATSUMOTO; YAMAHA, 1978).

Exposições dérmicas e pela via respiratória também são importantes rotas de exposição aos ftalatos, como DBP, que são usados em cosméticos (KOCH;

DREXLER; ANGERER, 2003; KOO; LEE, 2004). De acordo com Blount e colaboradores (2000), os níveis urinários de metabólitos do DBP são significativamente maiores em mulheres em idade reprodutiva (20 a 40 anos) quando comparado com as concentrações em homens ou em outras faixas etárias.

Em função da rápida metabolização, há pouca ou nenhuma bioacumulação. Um estudo revelou que uma semana após a exposição a uma dose única de DBP, nenhum tecido continha mais de 2% da dose administrada (ELSISI; CARTER; SIPES, 1989). A principal rota de excreção dos metabólitos do DBP é a urina (FOSTER *et al.*, 1983; ELSISI; CARTER; SIPES, 1989), sendo que ele é excretado após a conjugação com ácido glucorônico (LYCHE *et al.*, 2009). O MBP é excretado para a bile (cerca de 45%), mas apenas cerca de 5% é eliminado pelas fezes, indicando uma eficiente circulação êntero-hepática (TANAKA; MATSUMOTO; YAMAHA, 1978; ELSISI; CARTER; SIPES, 1989). A figura abaixo mostra o esquema metabólico do DBP em animais (FIGURA 03).

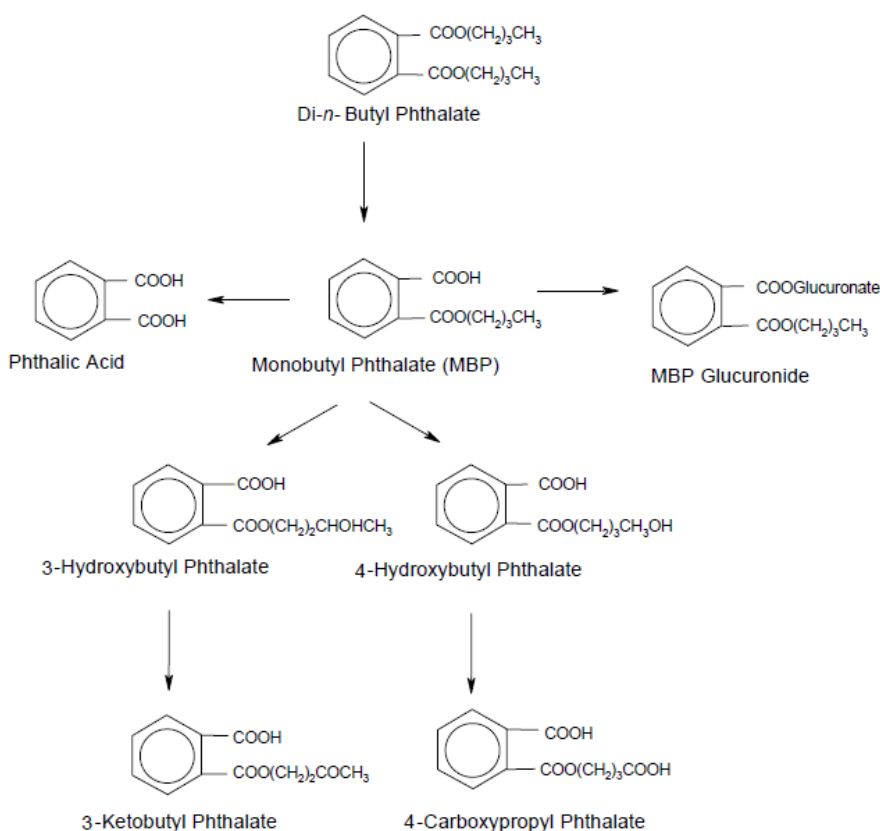


FIGURA 03. ESQUEMA METABÓLICO DO DIBUTIL FTALATO
FONTE: ATSDR (2001)

2.1.6 Aspectos Regulatórios dos Ftalatos

Regulamentações que restringem o uso de ftalatos vêm sendo desenvolvidas na tentativa de reduzir ou até mesmo evitar exposição humana direta a essas substâncias (KONIECKI *et al.*, 2011).

Na legislação brasileira, a Resolução nº 105 de 19 de maio de 1999 (ANVISA/MS) aprova, através do seu art.1º, os Regulamentos Técnicos - Disposições Gerais para Embalagens e Equipamentos Plásticos em contato com Alimentos e seus Anexos (BRASIL, 1999). Apesar de estar em vigor, o Anexo III desta lei, e seus Apêndices I e II, referente à Lista Positiva de Aditivos para Materiais Plásticos destinados à elaboração de Embalagens e Equipamentos em contato com Alimentos foram revogados pela RDC nº 17, de 17 de março de 2008 (BRASIL, 2008). Assim, os limites de ftalatos permitidos nas embalagens para alimentos estão definidos por esta Resolução (BRASIL, 2008). No Apêndice I do Anexo desta RDC consta que o DBP possui Limite de Migração Específica (LME) igual a 0,3 mg/kg, podendo ser usado como plastificante somente em materiais reutilizáveis que estejam em contato com alimentos não gordurosos; coadjuvante de tecnologia em poliolefinas em concentrações de até 0,05% no produto final (BRASIL, 2008).

Já a Portaria nº 369 de 27 de setembro de 2007 (INMETRO/ MDIC) estabelece, em seu art. 1º, os requisitos adicionais para ensaios toxicológicos em brinquedos e, em seu art. 2º os requisitos para aceitação e ensaio de ftalatos em brinquedos (BRASIL, 2007). No § 1º e 2º do art. 2º desta Portaria, contam que:

Art. 2º Estabelecer os requisitos para aceitação e ensaio de ftalatos em brinquedos.

§ 1º Os seguintes ftalatos: ftalato de di (2-etil-hexila) (DEHP), ftalato de dibutila (DBP), ftalato de benzilbutila (BBP) não devem ser utilizados, como substâncias ou componentes de preparações, em concentrações superiores a 0,1 % em massa de material plastificado, em todos os tipos de brinquedos de material vinílico.

§ 2º Os seguintes ftalatos: ftalato de di (2-etil-hexila) (DEHP), ftalato de dibutila (DBP), ftalato de benzilbutila (BBP), ftalato de di-isononila (DINP), ftalato de di-isodecila (DIDP) e ftalato de di-noctila (DNOP) não devem ser utilizados, como substâncias ou componentes de preparações, em concentrações superiores a 0,1 % em massa de material plastificado, em brinquedos de material vinílico destinados a crianças com idade inferior a 3 anos (BRASIL, 2007).

Além dessas regulamentações, está em análise pela Câmara dos Deputados, o Projeto de Lei nº 3222/12, o qual proíbe o uso da substância ftalato na composição de brinquedos e produtos destinados ao público infantil, tais como: chupetas, mamadeiras, roupas, calçados e material escolar (CAMARA DOS DEPUTADOS, 2012).

Já em relação ao uso de ftalatos em cosméticos, o Brasil ainda não possui nenhuma regulamentação, diferentemente da União Europeia que proibiu o uso de DBP e DEHP em cosméticos e produtos de higiene pessoal que participam do mercado europeu (EUROPEAN COMMISSION, 2007). No Brasil, a RDC nº 48 de 16 de março de 2006, a qual aprova o Regulamento Técnico sobre Lista de Substâncias que não podem ser utilizadas em Produtos de Higiene Pessoal, Cosméticos e Perfumes, não cita em sua listagem nenhum tipo de ftalato (BRASIL, 2006).

2.2 PROCESSOS ALÉRGICOS CUTÂNEOS

2.2.1 Estrutura da pele

A pele recobre a superfície do corpo e apresenta-se constituída por uma porção epitelial, a epiderme, e uma porção conjuntiva, a derme (FIGURA 04). A pele exerce diversas funções, principalmente proteção contra fatores químicos, físicos e biológicos, como a proteção contra raios ultravioleta através da melanina. Além disso, impede perda de água para o meio, promove as sensações de dor, pressão, tato e variações de temperatura, sintetiza hormônios (diidrotestosterona) e vitaminas (vitamina D), promove a regulação térmica, metaboliza xenobióticos e excreta substâncias através das glândulas sudoríparas (CHUONG *et al.*, 2002; HAAKE; SCOTT; HOLBROOK, 2000; SAMPAIO; CASTRO; RIVITTI, 2000; SHAEFER; REDELMEIER, 1996; ROSS; REITH; ROMRELL, 1993).

A epiderme é um tecido não vascular constituído por epitélio estratificado pavimentoso queratinizado, onde as células mais abundantes são os queratinócitos. A pele ainda apresenta outros tipos celulares como os melanócitos (que sintetizam a

melanina), as células de Langerhans (participam de reações imunes cutâneas) (GIROLOMONI *et al.*, 2002), as células de Merkel (mecano-receptoras) (KANITAKIS *et al.*, 1998), Linfócitos (<1,3%) (SPETZ; STROMINGER; GROH-SPIES, 1996), e as células de Toker (função ainda desconhecida, mas parecem expressar queratina) (LUNDQUIST; KOHLER; ROUSE, 1999).

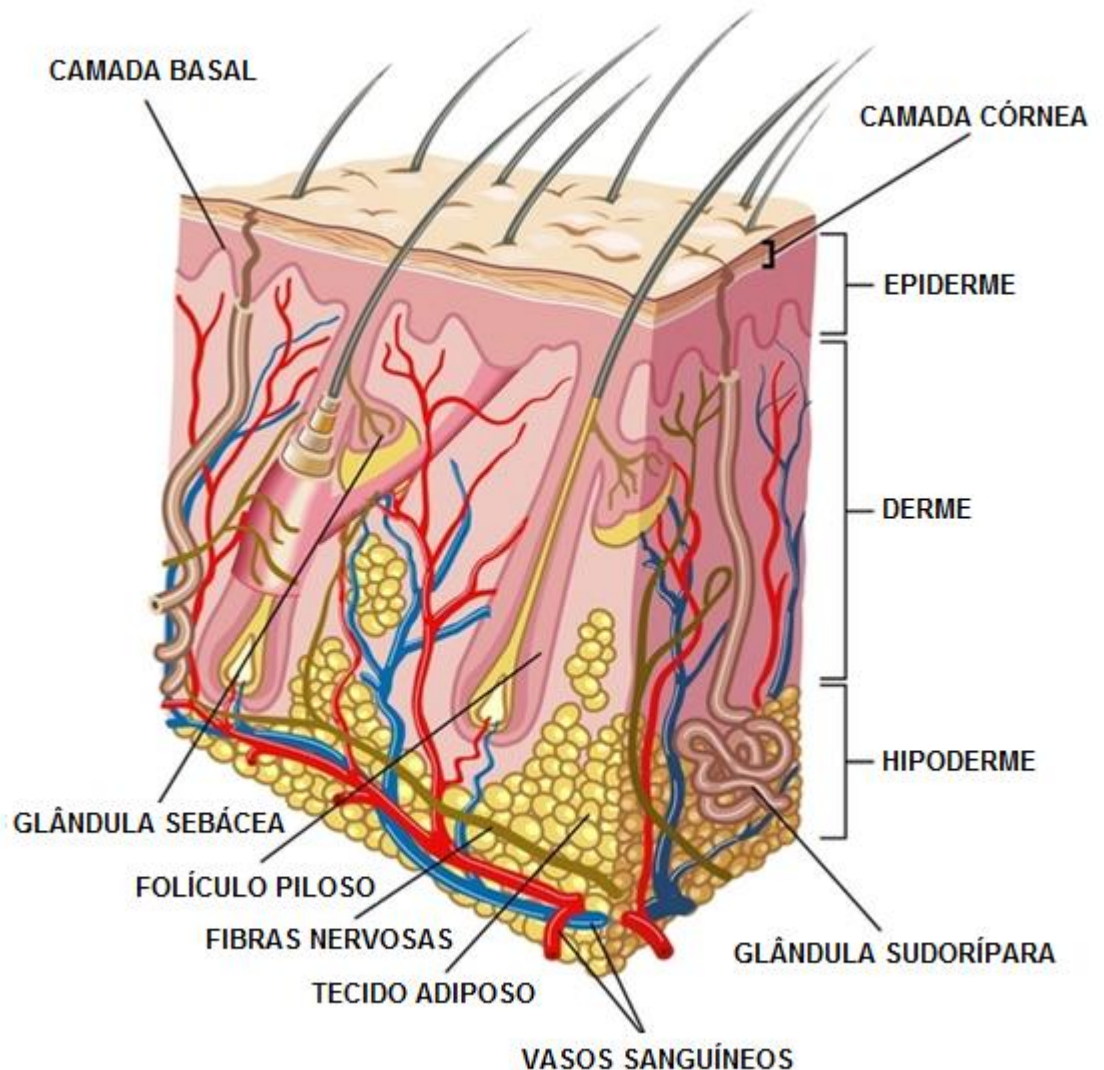


FIGURA 04 – ESTRUTURA DA PELE

FONTE: <http://www.livescience.com/27115-skin-facts-diseases-conditions.html>

Os queratinócitos representam o principal tipo celular presente na epiderme, sendo responsáveis pela manutenção da integridade da estrutura epidérmica. Também estão envolvidos na resposta imunológica do tecido cutâneo, uma vez que expressam diferentes citocinas, quimiocinas e também moléculas do complexo principal de histocompatibilidade da classe II (MHC-II). As células de Langerhans

estão localizadas nas camadas suprabasais da epiderme da pele e das mucosas, onde desempenham um importante papel na reposta imune cutânea (CHAN, 2004). As células de Langerhans são células migratórias que possuem vários receptores de membrana envolvidos no processo imunológico (ex.: MHC-II, IgG - imunoglobulina G, C3 - fator do complemento C3). Assim, essas células são responsáveis pelo reconhecimento, captação, processamento e apresentação de antígenos solúveis e haptenos aos linfócitos T. Em certas doenças inflamatórias cutâneas, como na dermatite de contato e em processos alérgicos, verifica-se que as células de Langerhans se tornam mais abundantes (NORRIS, 2004; HAAKE; SCOTT; HOLBROOK, 2000).

Além das células residentes da epiderme (queratinócitos, melanócitos, células de Langerhans e células de Merkel), outras células migram para a epiderme em resposta aos mais variados estímulos: linfócitos, macrófagos, neutrófilos e eosinófilos, sendo essas células elementos da resposta de defesa inata ou adquirida (NORRIS, 2004).

A derme é o tecido conjuntivo compressivo e elástico onde se apóia a epiderme e une a pele à hipoderme. A derme apresenta em sua constituição muitas fibras, sendo a maioria delas, as fibras de colágeno, principalmente dos tipos I e III, responsáveis pela resistência mecânica da pele. O tipo celular responsável pela síntese de fibras, presente na derme, é o fibroblasto (EYDEN, 2001). Os fibroblastos sintetizam diferentes macromoléculas que entram na constituição da matriz celular como, por exemplo, o colágeno e a elastina (HAAKE; SCOTT; HOLBROOK, 2000; SAMPAIO; CASTRO; RIVITTI, 2000; ROSS; REITH; ROMRELL, 1993). Durante um processo inflamatório ocorre o aumento da proliferação e da atividade de fibroblastos devido à ação de alguns mediadores pró-inflamatórios como a interleucina-1 α (IL-1 α) e interleucina-1 β (IL-1 β) (FREINKEL; WOODLEY, 2001).

Como a epiderme é um tecido não vascular, na derme é onde se encontra uma grande rede vascular responsável pela distribuição de nutrientes, cura de lesões, reações imunes e controle da pressão arterial (BRAVERMAN, 2000). Os vasos sanguíneos presentes na derme permitem que ocorra a infiltração de células migratórias importantes no processo de resposta de defesa inata ou imune e de cicatrização, como os macrófagos, linfócitos, eosinófilos, neutrófilos, entre outros (RYAN, 2004). A interação coordenada entre os diferentes tipos celulares presentes nas camadas da pele permite que este órgão responda, prontamente e

efetivamente, a uma variedade de estímulos nocivos que ocorrem na interface do organismo com o meio externo, como a ação de toxinas, organismos patogênicos, radiação ultravioleta, extremos de temperatura, garantindo assim a manutenção da homeostasia cutânea (BURBACH; ANSEL; ARMSTRONG, 2000; HAAKE; SCOTT; HOLBROOK, 2000; WILLIAMS; KUPPER, 1996). Nesse contexto, a pele é muito mais do que simplesmente uma barreira física passiva entre o meio externo e interno, mas também uma extensão do sistema imunológico (WILLIAMS; KUPPER, 1996).

2.2.2 Dermatite de contato alérgica (DCA) ou Hipersensibilidade de Contato (HSC)

Sabe-se que a prevalência de doenças alérgicas, como a dermatite atópica, asma brônquica e rinite alérgica vem aumentando (SEGAWA; HIRASAWA, 2014). A severidade e a indução de doenças alérgicas estão associadas a fatores genéticos e ambientais. Entre os fatores ambientais, a poluição do ar e os contaminantes químicos ambientais são considerados responsáveis pela exacerbação dessas doenças (SEGAWA; HIRASAWA, 2014). Um exemplo pode ser os ftalatos, que são considerados contaminantes onipresentes em alimentos, ar interno, solos e sedimentos (SHEA; COMMITTEE ON ENVIRONMENTAL HEALTH, 2003).

A dermatite de contato é uma dermatose inflamatória frequente nos países industrializados, sendo uma das doenças ocupacionais mais comuns. De acordo com os mecanismos fisiopatológicos envolvidos, podem-se distinguir dois tipos de dermatite de contato: a dermatite de contato irritativa, decorrente dos efeitos tóxicos e pró-inflamatórios de xenobióticos capazes de ativar a imunidade inata da pele; e a dermatite de contato alérgica, que requer a ativação da imunidade adquirida, levando ao desenvolvimento de células T efectoras, que são mediadoras da inflamação cutânea (HENNINO *et al.*, 2005). É caracterizada por eritema, pápulas e vesículas, seguidas de ressecamento e descamação.

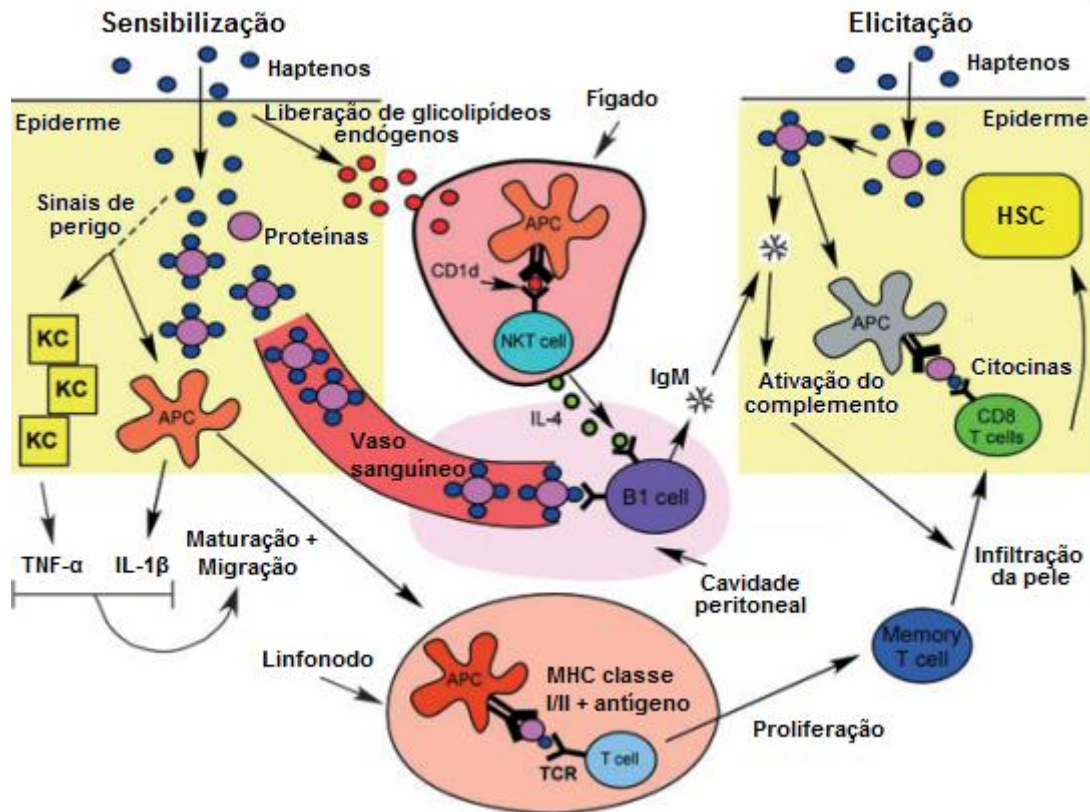
A dermatite de contato alérgica (DCA), também conhecida como hipersensibilidade de contato (HSC), é uma reação inflamatória cutânea mediada por células T decorrente de contatos repetidos da pele com substâncias químicas não protéicas, denominadas haptenos (SAINT-MEZARD *et al.*, 2004; LEPOITTEVIN;

LEBLOND, 1997). A iniciação da HSC é gerada pela aplicação tópica de haptenos sensibilizantes na epiderme. Duas fases são geralmente necessárias para atingir uma reação de HSC máxima: as fases de sensibilização e de indução.

A fase de sensibilização ocorre ao primeiro contato da pele com o hapteno e leva ao "priming" e a expansão de células T hapteno-específicas nos linfonodos. O hapteno aplicado topicamente é captado pelas células dendríticas (CD) cutâneas, especialmente as células de Langerhans (CL), que migram da epiderme para os linfonodos de drenagem, onde apresentam complexos de moléculas de peptídeo conjugado a hapteno aos precursores de células T hapteno-específicas. Células T específicas emigram dos linfonodos e atingem o sangue e recirculam no sangue e órgãos linfóides secundários. A fase de elicitação ou indução ocorre algumas horas após um contato subsequente da pele com o mesmo hapteno, que induz a produção de quimiocinas, a ativação de células endoteliais e mastócitos, e a infiltração de neutrófilos, todos necessários para o recrutamento de células T específicas. As células T interagem com células cutâneas apresentadoras de antígeno portadoras de hapteno. As células T CD8+ citotóxicas ativadas produzem citocinas tipo 1 e induzem a ativação de células cutâneas e a apoptose dos queratinócitos, levando a amplificação da inflamação cutânea através da produção de todo um conjunto de citocinas e quimiocinas. Esta fase de indução da HSC dura 72 horas em humanos e 24 a 48 horas em camundongos. A reação inflamatória persiste durante vários dias e diminui progressivamente mediante mecanismos de regulação negativa fisiológicos (FIGURA 05).

Além das células T, outras subpopulações de células linfóides contribuem para a complexa via que finalmente leva a resposta de HSC (ASKENASE, 2001; YOKOZEKI *et al.*, 2001). As células B-1 são ativadas nos órgãos linfóides dentro de horas após a sensibilização cutânea e produzem anticorpos IgM. Esses anticorpos difundem-se na pele e ligam-se ao hapteno imediatamente após o desafio. A presença de complexos imunes dá início a ativação do complemento, que parece mandatória para o recrutamento de células T efetoras no local do desafio (CAMPOS *et al.*, 2003). Os neutrófilos desempenham um importante papel no desenvolvimento da HSC. Em sua ausência a HSC é reduzida. Segundo a literatura, estão envolvidos nas fases de sensibilização e elicitação da HSC. Os neutrófilos estão entre as primeiras células a serem recrutadas após o desafio de camundongos sensibilizados (DILULIO *et al.*, 1999) e aparecem antes da infiltração de células T CD8 efetoras.

Uma vez que as células efetoras tenham sido ativadas, outro influxo de neutrófilos é secundário a ativação de mastócitos que produzem $\text{TNF-}\alpha$ (BIEDERMANN *et al.*, 1999).



Fases de sensibilização e elicitação da hipersensibilidade de contato. Durante a sensibilização, haptenos em contato com a pele interagem com proteínas epidérmicas para formar complexos hapteno-proteína. Estes são capturados pelas células apresentadoras de antígenos (APCs, células dendríticas e de Langerhans). Além disso, o contato do hapteno com a pele induz um sinal de perigo que ativa queratinócitos (KC) que então liberam citocinas como $\text{TNF-}\alpha$ e $\text{IL-1}\beta$. Com isso, há a maturação de células apresentadoras de antígenos e sua migração aos linfonodos. Nos linfonodos, células apresentadoras de antígenos ativam células T antígeno-específicas, que se tornam células T de memória. O contato da pele com o hapteno também leva à liberação de glicolídeos endógenos que são apresentados pelas células apresentadoras de antígenos a células NKT no fígado levando à liberação de IL-4 . Simultaneamente, complexos hapteno-proteína são liberados do local de sensibilização para a circulação e transferidos para a cavidade peritoneal. Na presença de IL-4 e complexos hapteno-proteína, células B-1 na cavidade peritoneal são ativadas e migram para o baço e linfonodos onde liberam IgM. Na fase de elicitação, IgM interage com o complexo hapteno-proteína e induz a ativação do complemento, que leva à liberação de mediadores inflamatórios e quimiotáticos dos mastócitos e células endoteliais. Finalmente, células T CD8 antígeno-específicas migram para o local da elicitação e interagem com as células apresentadoras de antígeno resultando em liberação de citocinas inflamatórias e levando ao desenvolvimento dos sintomas da hipersensibilidade de contato (HSC).

FIGURA 05 - FISIOPATOLOGIA DA DERMATITE DE CONTATO ALÉRGICA

FONTE: Christensen e Haase (2012) – adaptado.

Dentre as citocinas, destacam-se as citocinas pró-inflamatórias IL-1 β e TNF- α (LI; CRUZ, 2004; ENK, 1997). A IL-1 β liberada pelas células de Langerhans faz com que os queratinócitos produzam TNF- α que, juntamente com a IL-1 β , determinam a maturação e migração dessas CD para os linfonodos (ENK *et al.*, 1993; STEINMAN; HOFFMAN; POPE, 1995). IL-8, liberada por células dendríticas expostas a alérgenos de contato, tem um papel importante na sensibilização e na eliciação de reações alérgicas, por induzir o influxo de leucócitos para o local da inflamação. IL-8 é um potente atrativo de neutrófilos e diferentes subtipos de células T (BARKER *et al.*, 1991). Além de IL-8, IL-18, expressa por queratinócitos após exposição à alérgenos de contato (NAIK *et al.*, 1999; VAN OCH *et al.*, 2005), é importante na indução da hipersensibilidade de contato por aumentar a secreção de mediadores pró-inflamatórios como TNF α , IL-8 and IFN- γ (OKAMURA *et al.*, 1995; CUMBERBATCH *et al.*, 2001; ANTONOPOULOS *et al.*, 2008).

2.2.3 Papel do estresse oxidativo na hipersensibilidade de contato

Evidências indicam que doenças cutâneas inflamatórias e alérgicas possam ser mediadas por estresse oxidativo (OKAYAMA, 2005; BYAMBA *et al.*, 2010). Análises genômicas e proteômicas de queratinócitos humanos assim como de células dendríticas revelaram um grande número de marcadores específicos de sensibilizantes com diversas sobreposições interessantes.

No sistema imune, espécies reativas de oxigênio estimulam a resposta imune através da ativação de vias de sinalização, aumento da expressão de moléculas co-estimulatórias, carbonilação de proteínas e secreção de citocinas (LARBI *et al.*, 2007; SAREILA *et al.*, 2011; BERTOLOTTI; SITIA; RUBARTELLI, 2012). Recentemente, vários estudos apóiam a participação de espécies reativas de oxigênio como o gatilho da dermatite de contato alérgica (GLOIRE; LEGRAND-POELS; PIETTE, 2006; KIM *et al.*, 2012), outros propõem que um dos papéis biológicos da dermatite de contato seja uma defesa contra substâncias químicas com potencial de alterar o balanço redox das células (SASAKI; AIBA, 2007).

A habilidade de substâncias sensibilizantes de induzir estresse oxidativo em queratinócitos (NATSCH; EMTER, 2007) foi recentemente confirmada por

Vandebriel, Van Och e Van Loveren (2010). Além disso, já foi demonstrado que a produção de IL-18 induzida por alérgenos em queratinócitos requer a produção de espécies reativas de oxigênio e é prevenida pelo antioxidante pirrolidina ditiocarbamato (PDTC) (GALBIATI *et al.*, 2011; CORSINI *et al.*, 2009).

Em células dendríticas, Mizuashi *et al.* (2005) mostraram que todas as substâncias sensibilizantes testadas (níquel, formaldeído, DNCB, cloreto de manganês e timerosal), mas nenhuma das substâncias não sensibilizantes reduziu a razão entre as formas reduzida e oxidada da glutathione (GSH/GSSG), o que foi acompanhado de ativação da proteína quinase ativada por mitógeno (MAPK) p38, um marcador da maturação de células dendríticas induzida por sensibilizantes químicos. O antioxidante N-acetil-L-cisteína foi capaz de suprimir a redução da razão GSH/GSSG e revogar a ativação da p38 MAPK.

2.2.4 Influências do sistema nervoso periférico na HSC

O sistema nervoso cutâneo representa uma parte do sistema nervoso periférico (BERESFORD *et al.*, 2004; LIU *et al.*, 2006). Anatomicamente, células de Langerhans estão associadas a terminações nervosas (BERESFORD *et al.*, 2004).

Neuropeptídeos na pele são sintetizados e liberados predominantemente por uma subpopulação de neurônios aferentes não-mielinizados chamados de nociceptores polimodais C, que representam 70% de todas as fibras C cutâneas (LAWSON, 1996). Essas fibras C expressam receptores sensíveis a capsaicina, chamados TRPV1 (BÁNVÖLGYI *et al.*, 2005; MURAI *et al.*, 2008; RAZAABI *et al.*, 2006). Alguns dos neurônios sensoriais que expressam TRPV1 também expressam TRPA1 (STORY *et al.*, 2003), ativados por cinamaldeído, alil isotiocianato, dentre outros estímulos (BAUTISTA *et al.*, 2005). A ativação de TRPA1 ou TRPV1 em terminações nervosas sensoriais resulta na liberação de neuropeptídeos como o peptídeo relacionado ao gene da calcitonina (CGRP) (BAUTISTA *et al.*, 2005; ZYGMUNT *et al.*, 1999). Desde que o tratamento de humanos ou cobaias (*Cavia porcellus*) com capsaicina gerou um aumento na intensidade da resposta de hipersensibilidade de contato no local do tratamento, foi sugerido que neurônios

sensíveis a capsaicina poderiam modular a HSC através da liberação de neuropeptídeos (GIROLOMONI; TIGELAAR, 1990; EK; THEODORSSON, 1990).

Os receptores de potencial transitório (TRPs) constituem uma superfamília de receptores que contribuem para mudanças nas concentrações intracelulares de Ca^{2+} , e que tem papel fundamental em diversos processos celulares como contração muscular, liberação de transmissores e morte celular (BERRIDGE; LIPP; BOOTMAN, 2000). Essa superfamília é subdividida em sete subfamílias: TRPC, TRPM, TRPV, TRPA, TRPP, TRPML, TRPN (MONTELL; BIRNBAUMER; FLOCKERZI, 2002; COREY, 2003; CLAPHAM, 2003; MORAN; XU; CLAPHAM, 2004).

A subfamília vanilóide, ou TRPV, consiste em quatro grupos nos mamíferos: TRPV1/V2, TRPV3, TRPV4 e TRPV5/V6 (GUNTHORPE *et al.*, 2002; BENHAM; DAVIS; RANDALL, 2002). O TRPV1 foi o primeiro dessa família a ser identificado em mamíferos e é um dos mais estudados. TRPV1 são altamente expressos em terminações nervosas periféricas (PLANELLIS-CASES *et al.*, 2005) distribuídas na derme, além de serem expressos em queratinócitos e células dendríticas (GOPINATH *et al.*, 2007; BASU; SRIVASTAVA, 2005) e são ativados por compostos vanilóides, como a capsaicina, e por outros estímulos como calor moderado, pH baixo e outros compostos (MONTELL; BIRNBAUMER; FLOCKERZI, 2002; PLANELLIS-CASES *et al.*, 2005). A capsaicina não ativa nenhum outro receptor da mesma família (PEDERSEN; OWSIANIK; NILIUS, 2005). Alguns estudos sugerem que a ativação de TRPV1 em neurônios sensoriais possa ter influências sobre o sistema imune (BASU; SRIVASTAVA, 2005; BÁNVÖLGYI *et al.*, 2005; MURAI *et al.*, 2008; RAZAAVI *et al.*, 2006).

Já a subfamília anquirina, ou TRPA, apresenta somente um membro conhecido em mamíferos, o TRPA1. O TRPA1 é ativado por isotiocianatos e cinamaldeído, entre outros compostos, mas é insensível a capsaicina (PEDERSEN; OWSIANIK; NILIUS, 2005). Estudos já demonstraram que a ativação de receptores TRPA1 na pele participa do desenvolvimento de respostas inflamatórias desenvolvidas pela aplicação tópica de um agonista TRPA1 (SILVA *et al.*, 2011) e que estes receptores também são expressos em queratinócitos (ATOYAN; SHANDER; BOTCHKAREVA, 2009).

Sabe-se também que, quando estimuladas, as fibras C podem liberar uma série de neuropeptídeos que tem a capacidade de modular a resposta inflamatória

local. É conhecido que a ativação de receptores TRPA1 e TRPV1 em terminações nervosas sensoriais resulta na liberação de neuropeptídeos como o CGRP (peptídeo relacionado ao gene da calcitonina) e a substância P (SP) (BAUTISTA *et al.*, 2005; ZYGMUNT *et al.*, 1999).

A contribuição dos neuropeptídeos para inflamação neurogênica vem de estudos usando a capsaicina. A resposta aguda à aplicação tópica de capsaicina na orelha de camundongos é característica de inflamação neurogênica, onde o aumento do fluxo sanguíneo e formação de edema são dependentes da liberação e ação do CGRP e SP (GRANT, 2002). A SP é o mediador clássico da resposta: eritema, edema e prurido. SP e CGRP injetados em pele humana rapidamente induzem infiltração de eosinófilos e neutrófilos (SMITH *et al.*, 1993), sugerindo que esses neuropeptídeos participam do recrutamento leucocitário. A SP também pode modular o padrão de expressão de citocinas pelos queratinócitos e mastócitos, aumentando a produção de citocinas pró-inflamatórias como IL-1 e IL-8 (BROWN *et al.*, 1990; VIAC *et al.*, 1996) e induzindo a expressão do mRNA e proteína para TNF- α (ANSEL *et al.*, 1993) em cobaias. Sumarizando, a SP tem um papel direto ou indireto aumentando a inflamação neurogênica cutânea. CGRP é um dos neuropeptídeos mais abundantes na pele e é frequentemente colocalizado com a SP (GIBBINS; WATTCHOW; COVENTRY, 1987). Um dos efeitos mais proeminentes do CGRP *in vivo* são ações vasodilatadoras. Injeção intradérmica de CGRP causa eritema de longa duração (JANSEN-OLESEN; MORTENSEN; EDVINSSON, 1996; BRAIN *et al.*, 1986). O CGRP potencializa a formação de edema causado por neuropeptídeos como a SP, (SCHOLZEN *et al.*, 1998). Além disso, o CGRP também pode prejudicar a apresentação de antígeno necessária nas reações de HSC através do aumento da produção de citocinas pró-inflamatórias como IL-10, que pode então inibir ações das células T (TORII *et al.*, 1997).

2.3 ABSORÇÃO E TOXICIDADE DÉRMICA DOS FTALATOS

Estudos recentes indicam que os ftalatos, em especial o DBP, são capazes de serem absorvidos pela pele. Elsisí, Carter e Sipes (1989) identificaram que após sete dias da aplicação tópica de DBP, cerca de 50 a 60% da dose foi excretada,

sendo a via urinária a principal via de excreção, ou seja, eles não são bioacumulados. Além disso, identificaram que 33 ± 2 % da dose aplicada permaneceu no local de aplicação. Segundo os autores, a extensão da absorção tópica dos ftalatos pode estar associada a fatores que competem entre si, tais como lipofilia, peso molecular e metabolismo.

Janjua e colaboradores (2008) identificaram e dosaram DBP e seus metabólitos na urina após aplicação tópica em humanos e, Sathyanarayana *et al.* (2008) demonstraram que mais de sete metabólitos de ftalatos foram detectados na urina de 81% de crianças após o uso de produtos infantis. Em outro estudo, testes *in vivo* (em cobaias) e testes *in vitro* mostram que o DBP inserido em uma formulação (emulsão óleo/água) pode ser absorvido pela pele e ficar potencialmente disponível para distribuição sistêmica (DOAN; BRONAUGH; YOURICK, 2010).

A absorção dérmica do DBP em ratos parece estar associada à atividade de esterases, que o hidrolisam durante a absorção percutânea (BEYDON; PAYAN; GRANDCLAUDE, 2009).

Em relação à toxicidade do DBP, uma irritação leve foi observada em estudos em pele de coelhos usando uma dose de 520 mg/kg, uma dose extremamente alta para exposição cutânea (NATIONAL TOXICOLOGY PROGRAM, 2003). Testes agudos e crônicos realizados pela *Consumer Product Safety Commission* levaram a conclusão de que existe pouca evidência em humanos e animais para designar o DBP como substância sensibilizante de acordo com os critérios propostos pela Consumer Product Safety Commission (CPSC, 2010).

Em estudos crônicos realizados em cobaias e em estudos agudos em coelhos não foi encontrada sensibilização causada pelo DBP (NICNAS, 2008). Em humanos, os dados são inconclusivos. Alguns casos isolados foram reportados em humanos. Duas mulheres desenvolveram dermatite após o uso de um desodorante contendo DBP. As duas apresentaram resposta positiva para um teste de contato contendo DBP, mas apresentaram respostas negativas para outros constituintes do desodorante (NICNAS, 2008).

Chowdhury e Statham (2002) reportaram um caso de um homem de 65 anos que desenvolveu hipersensibilidade de contato após usar uma pomada para prurido. O indivíduo também apresentou resposta positiva para o teste de contato contendo DBP. Mas, em um estudo que expôs 159 indivíduos a um teste de contato contendo cosméticos que apresentavam uma concentração de DBP de 4,5 a 9%, nenhum

indivíduo apresentou resposta positiva. Mais recentemente, Pan *et al.* (2014) observaram que após aplicação dérmica do DBP, houve apoptose de queratinócitos e fibroblastos via ativação da via de caspases -3.

Por fim, deve-se ressaltar a importância da exposição ocupacional aos ftalatos. Em seu estudo, Kwapniewski *et al.* (2008) dosou MBP (monobutil ftalato), principal metabólito do DBP, em urina de manicures e, observou um aumento de 17,4 ng/mL desse metabólito durante o turno de trabalho, sendo que o uso de luvas reduziu esse valor para 15,1 ng/mL. Todos esses estudos em conjunto indicam que a toxicidade dérmica dos ftalatos deve ser melhor avaliada.

2.3.1 Ftalatos e processos alérgicos

Há relatos de que alguns ésteres de ftalato estão associados a sintomas alérgicos em crianças (BORNEHAG *et al.*, 2004; KOLARIK *et al.*, 2008), bem como em adultos (CHOWDHURY; STATHAM, 2002). Latini e colaboradores (2003) propuseram um papel dos ftalatos na indução de inflamação intrauterina devido a uma semelhança estrutural entre os ésteres de ftalato e algumas prostaglandinas e tromboxanas pró-inflamatórias. Scarano e colaboradores (2009) mostraram a presença de focos de inflamação na próstata de ratos expostos *in utero* e durante a lactação a 100 mg/kg/dia de DBP.

Em relação a processos alérgicos na pele, alguns estudos demonstram que certos ésteres de ftalatos, como o DBP, não possuem um potencial significativo de causarem sozinhos a sensibilização da pele e dermatites de contato alérgicas. Isto porque essas moléculas não são capazes de se ligarem e serem reativas a proteínas, pré-requisito para a estimulação alérgica por uma substância química (KIMBER; DEARMAN, 2010). Porém, Chowdhury e Statham (2002) reportaram casos de hipersensibilidade de contato induzidas pelo DBP. Além disso, Imai e colaboradores (2006) observaram que durante o processo de sensibilização por isotiociano de fluoresceína (FITC), o DBP exerceu forte efeito adjuvante, associado ao aumento do tráfico de células dendríticas apresentadoras de antígenos a partir da pele para os linfonodos de drenagem.

Matsuda *et al.* (2010) encontrou que o DBP pode aumentar a sensibilização da pele quando associado a alguns haptenos como, o feniletil isocianato (PEITC), além de dois análogos do FITC (isotiocianato de eosina-5 e isotiocianato de rodamina B). Porém, não é capaz de exacerbar a sensibilização quando associado à oxazolona e 2,4-dinitrofluorobenzeno (que exibem uma resposta mediada por linfócitos Th-1) ou ao anidrido trimelítico, metilenodifenilo 4,4-di-isocianato e 2,4-diisocianato de tolueno (que exibem uma resposta mediada por linfócitos Th-2).

Em estudo mais recente, Li *et al.* (2014), utilizando o modelo de hipersensibilidade por contato induzido por FITC, investigou a exposição prolongada (40 dias) ao DBP em doses de 0,4, 4,0 e 40 mg/kg/dia. Os resultados mostraram que essa exposição pode agravar a dermatite de contato alérgica em camundongos, havendo uma reação linear entre dose-resposta, com aumento do edema, na contagem de células infiltradas, entre outros parâmetros.

2.4 MODELOS DE HIPERSENSIBILIDADE DE CONTATO

Modelos de hipersensibilidade de contato em camundongo vêm sendo muito utilizados, a fim de se obter informações sobre a apresentação de antígenos, bem como a ativação de linfócitos T. Esses modelos são amplamente difundidos para estudo da inflamação mediada pelo sistema imune, devido à facilidade de aplicação da substância sensibilizante, medida da resposta, amostragem e acesso aos linfonodos de drenagem (WEBB *et al.*, 1998). Alguns modelos animais de inflamação cutânea auxiliam a identificar substâncias sensibilizantes, pois promovem condições que se assemelham com alguns tipos de dermatites observadas em humanos (VANE; BOTTING, 1998; BOUCLIER *et al.*, 1990).

Dentre as substâncias sensibilizantes, a oxazolona é frequentemente utilizada por ser capaz de induzir as duas fases da hipersensibilidade de contato. A oxazolona é considerada um hapteno forte, com propriedades pró-inflamatórias potentes (ZHANG; TINKLE, 2000). A oxazolona é um potente agente sensibilizante que produz pouca irritação. Por ser capaz de produzir uma inflamação crônica, dependente de linfócitos T tipo 1 (Th1) na fase inicial, e tipo 2 (Th2) seguindo exposição continuada do agente (WEBB *et al.*, 1998), e de ser de fácil

reprodutibilidade, a hipersensibilidade de contato induzida por oxazolona (FUJII *et al.*, 2002) tem se mostrado um modelo farmacologicamente útil nos estudos de dermatite de contato alérgica, já que reproduz os aspectos dessa doença humana, induzindo elevação nos níveis de IFN- γ e pronunciável hiperplasia epidérmica (BONISH *et al.*, 2000). O modelo de edema de orelha induzido pela oxazolona (FUJII *et al.*, 2002) pode ser utilizado para identificar substâncias sensibilizantes baseado na capacidade de aumento do espessamento da orelha de animais sensibilizados (KIMBER *et al.*, 1999), que vem acompanhado de infiltração celular e liberação de diversas citocinas (WEBB *et al.*, 1998; FUJII *et al.*, 2002; BAS *et al.*, 2007).

No entanto, seria importante tanto do ponto de vista econômico quanto ético, desvendar marcadores biológicos que sejam detectáveis *in vitro* para discriminar substâncias sensibilizantes as quais os humanos estejam expostos. Na última década, grande progresso tem sido alcançado no desenvolvimento de testes *in vitro* alternativos para substituir ou complementar os testes em animais.

Corsini *et al.* (2009) propuseram um ensaio *in vitro* baseado na produção de interleucina 18 (IL-18) na linhagem queratinocítica humana NCTC 2544 para identificar sensibilizantes de contato e distingui-los de irritantes e alérgenos respiratórios (GALBIATI *et al.*, 2011; CORSINI *et al.*, 2013a). IL-18 favorece a resposta imune do tipo Th-1 aumentando a secreção de mediadores pró-inflamatórios como TNF- α , IL-8 e IFN- γ (CUMBERBATCH *et al.*, 2001).

Mitjans *et al.* (2008) reportaram na linhagem celular monocítica THP-1 uma produção seletiva da quimiocina interleucina 8 (IL-8) por alérgenos de contato em paralelo a um aumento na expressão da proteína de membrana CD86 (cluster de diferenciação 86) e propuseram esse ensaio como um possível teste *in vitro* de sensibilização. Esse ensaio é utilizado como um substituto de ativação de células dendríticas, já que a expressão de CD86 ocorre durante a maturação de células dendríticas (QUAH; O'NEILL, 2005).

3 HIPÓTESES E PREDIÇÕES

Propomos que ao associarmos o dibutil ftalato a alérgenos em testes *in vitro* e *in vivo* de hipersensibilidade de contato, possamos identificar um efeito adjuvante do DBP. Se essa hipótese for verdadeira, esperamos observar uma exacerbação na resposta inflamatória envolvida no modelo *in vivo*, levando a aumento de edema e do recrutamento celular; ou no aumento da expressão e liberação de marcadores de ativação celular nos ensaios *in vitro*. Além disso, acreditamos que o estresse oxidativo e ativação de receptores TRPA1 e TRPV1 sejam parte do mecanismo molecular envolvido no efeito adjuvante do DBP.

4 OBJETIVOS

4.1 OBJETIVO GERAL

Investigar o efeito adjuvante do plastificante DBP em modelos *in vivo* e *in vitro* de HSC, caracterizando o perfil de células envolvidas nesses modelos, bem como a determinação do possível papel do estresse oxidativo e dos receptores TRPV1 e TRPA1 nesse efeito.

4.2 OBJETIVOS ESPECÍFICOS

- Investigar o potencial do DBP como sensibilizante de contato em dois testes *in vitro* usando linhagens celulares humanas, o ensaio NCTC 2544 e o ensaio de ativação de células THP-1.

- Investigar o possível efeito adjuvante do DBP pela avaliação da expressão de CD86 e IL-8 mRNA e liberação de IL-8 em células THP-1 após exposição dos alérgenos oxazolona, citral e imidazolidinil ureia associados ao DBP.

- Investigar o possível efeito adjuvante do DBP pela avaliação da liberação de IL-18 em células NCTC 2544 após exposição ao alérgeno p-Fenilenodiamina associado ao DBP.

- Avaliar o potencial adjuvante do DBP sobre a HSC induzida em camundongos pela oxazolona.

- Avaliar o possível papel indutor do DBP (desafio) em camundongos sensibilizados com oxazolona.

- Verificar a participação da indução de estresse oxidativo no efeito adjuvante do DBP na HSC induzida por oxazolona.

- Avaliar a participação dos receptores TRPA1 e TRPV1 na ação adjuvante do DBP na HSC.

-Analisar a infiltração de leucócitos, edema e espessura da epiderme por meio de análise histológica do tecido auricular.

-Avaliar o perfil de células inflamatórias envolvidas no efeito adjuvante do DBP no modelo de HSC induzida por oxazolona.

5 MATERIAIS, MÉTODOS E RESULTADOS

5.1 CONSIDERAÇÕES GERAIS


Os procedimentos experimentais *in vivo* do presente estudo foram realizados de acordo com princípios éticos, e aprovados pela Comissão de Ética no Uso de Animais do Setor de Ciências Biológicas da Universidade Federal do Paraná (CEUA/BIO nº 633).

O material e metodologia empregados, bem como resultados e discussão que compõe a presente tese estão descritos nos manuscritos apresentados a seguir (artigos 1, 2 e 3). Ao final de cada artigo encontra-se incluído seu respectivo material complementar para melhor visualização dos resultados.

5.2 ARTIGO 1

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The plasticizer dibutyl phthalate (DBP) potentiates chemical allergen-induced THP-1 activation

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ABSTRACT

Recent studies suggest that phthalates may have a role in the development of allergic diseases, probably due to an adjuvant effect. The present study aimed to investigate the possible adjuvant effect of dibutyl phthalate (DBP) in two in vitro models of contact-allergen induced cell activation, namely the NCTC 2544 IL-18 assay and the THP-1 activation assay. Results show no adjuvant effect in the human keratinocyte cell line NCTC 2544, indicated by lack of increase in interleukin 18 (IL-18) production after exposure to p-Phenylenediamine (PPD) in association with DBP. On the contrary, increased upregulation of CD86 and interleukin 8 (IL-8) production were observed in THP-1 cells exposed to combinations of citral (Cit) or imidazolidinyl urea (IMZ) with DBP, indicative of an adjuvant effect. Additionally, higher production of reactive oxygen species (ROS) in THP-1 cells treated with DBP associated to Cit supports that oxidative stress could be part of the molecular mechanism of the observed adjuvant effect. In conclusion, we demonstrate that DBP presents in vitro an adjuvant effect for immune stimulation in dendritic cells but not in keratinocytes. Future studies are necessary to elucidate the precise mechanism underlying the adjuvant effect of DBP in vitro and in vivo.

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The plasticizer dibutyl phthalate (DBP) potentiates chemical allergen-induced THP-1 activation

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ABSTRACT

Recent studies suggest that phthalates may have a role in the development of allergic diseases, probably due to an adjuvant effect. The present study aimed to investigate the possible adjuvant effect of dibutyl phthalate (DBP) in two *in vitro* models of contact-allergen induced cell activation, namely the NCTC 2544 IL-18 assay and the THP-1 activation assay. Results show no adjuvant effect in the human keratinocyte cell line NCTC 2544, indicated by lack of increase in interleukin 18 (IL-18) production after exposure to p-Phenylenediamine (PPD) in association with DBP. On the contrary, increased upregulation of CD86 and interleukin 8 (IL-8) production were observed in THP-1 cells exposed to combinations of citral (Cit) or imidazolidinyl urea (IMZ) with DBP, indicative of an adjuvant effect. Additionally, higher production of reactive oxygen species (ROS) in THP-1 cells treated with DBP associated to Cit supports that oxidative stress could be part of the molecular mechanism of the observed adjuvant effect. In conclusion, we demonstrate that DBP presents *in vitro* an adjuvant effect for immune stimulation in dendritic cells but not in keratinocytes. Future studies are necessary to elucidate the precise mechanism underlying the adjuvant effect of DBP *in vitro* and *in vivo*.

Key words: dibutyl phthalate, adjuvant effect, contact dermatitis, keratinocytes, dendritic cells, *in vitro* toxicology

1 INTRODUCTION

Phthalate esters are widely used as plasticizers in a variety of products, including soft polyvinyl chloride toys, vinyl floorings, wall coverings, synthetic leather and blood transfusion bags. Due to their wide distribution in the environment, they are considered agents with high potential for human exposure (KOO; LEE, 2004; ANDERSON *et al.*, 2001; SILVA *et al.*, 2004; LYCHE *et al.*, 2009; JEONG *et al.*, 2011). Some phthalate esters with short alkyl chains, like dibutyl phthalate (DBP), are used in cosmetics and mosquito repellents for topical use (INT. J. TOXICOL., 2005; VARTAK; TUNGKAR; SHARMA, 1994). Besides, phthalates with lower molecular weights are more highly absorbed (ELSISI; CARTER; SIPES, 1989).

DBP, as well as certain other phthalates, has attracted great attention from the scientific community because of their possible endocrine disruptive effects that can alter the normal development of the male reproductive tract (FISHER *et al.*, 2003; HEUDORF; MERSCH-SUNDERMANN; ANGERER, 2007; MARTINO-ANDRADE *et al.*, 2009). Besides the reproductive and endocrine effects, experimental and epidemiological data also suggest that phthalates could play a role as a possible contributor in the development and/or enhancement of atopic allergic diseases, thus, possibly contributing to the increased prevalence of atopic IgE-mediated allergic diseases in industrialized countries (IMAI *et al.*, 2006; KOIKE *et al.*, 2010; MATSUDA *et al.*, 2010; LI *et al.*, 2014; BORNEHAG *et al.* 2004; JAAKKOLA; KNIGHT, 2004). It has been suggested that certain phthalates may act as adjuvants, meaning that they could accelerate, prolong or enhance a specific immune response (KIMBER; DEARMAN, 2010). However, no consistent pattern regarding to dose and route of exposure has emerged. Additional research is needed to better understand whether, to what extent, and under what conditions phthalates are able to effect a meaningful change in immune function that may in turn impact human health (KIMBER; DEARMAN, 2010).

Allergic contact dermatitis is a delayed type hypersensitivity reaction resulting from repeated contacts of the skin with low molecular weight chemicals called haptens (LEPOITTEVIN; LEBLOND, 1997; SAINT-MEZARD *et al.*, 2004). Several cell types are involved in the contact hypersensitivity response. Keratinocytes respond to allergens by producing a range of pro-inflammatory cytokines, which are

important for dendritic cell maturation and migration to the lymph node, where the activation of the specific immune response takes place (VANDEBRIEL; VAN OCH; VAN LOVEREN, 2005). Dendritic cells are recognized as important antigen presenting cells in adaptive immunity because of their capacity to stimulate naive lymphocytes (BANCHEREAU *et al.*, 2000).

Corsini *et al.* (2009) proposed an *in vitro* assay based on the interleukin 18 (IL-18) production in the human keratinocyte cell line NCTC 2544 to identify contact sensitizers and discriminate them from irritants and respiratory allergens (GALBIATI *et al.*, 2011; CORSINI *et al.*, 2013a). IL-18 has been demonstrated to favor Th-1 type immune response by enhancing the secretion of pro-inflammatory mediators such as TNF- α , IL-8 and IFN- γ , and to play a key proximal role in the induction of allergic contact dermatitis (CUMBERBATCH *et al.*, 2001). Mitjans *et al.* (2008) reported in the human promyelocytic cell line THP-1 (used as a surrogate of dendritic cells), a selective production of the chemokine interleukin 8 (IL-8) by contact allergens in parallel to the surface marker cluster of differentiation 86 (CD86) upregulation, and, proposed this as a possible *in vitro* sensitization test. IL-8 is a potent attractant for neutrophils and different subtypes of T cells (BARKER *et al.*, 1991).

Oxidative stress has been reported to be central to chemical allergen-induced contact dermatitis and inflammatory skin diseases (OKAYAMA, 2005; BYAMBA *et al.*, 2010; CORSINI *et al.*, 2013b). Several authors have reported a relationship between phthalates and oxidative stress demonstrating increasing generation of reactive oxygen species (ROS) (SEO *et al.*, 2004; TETZ *et al.*, 2013). Others have associated the presence of several phthalate monoester metabolites detected in urine, with increased serum markers of inflammation and oxidative stress (HAUSER *et al.*, 2007; FERGUSON; LOCH-CARUSO; MEEKER, 2011).

With this study, we aimed to investigate the mechanism underlying the possible adjuvant effect of DBP. We evaluated the effects of DBP alone or in association with selected chemical allergens in two *in vitro* models as representative of keratinocytes and dendritic cells responses, the human keratinocyte cell line NCTC 2544 and the human promyelocytic cell line THP-1, respectively. We also investigated if the generation of ROS could be part of the molecular mechanism of the adjuvant effect induced by DBP. Even though several studies have been conducted in animals to evaluate the adjuvant effect of phthalates, little can be found about this effect *in vitro*.

2 MATERIAL AND METHODS

2.1 CHEMICALS

p-Phenylenediamine (PPD), citral (Cit) and imidazolidinyl urea (IMZ) were selected as skin sensitizers. Allergens and dibutyl phthalate (DBP) were purchased from Sigma (St. Louis, MO, USA) at the highest purity available (PPD 99%, Cit 95%, IMZ 95%, DBP 99%). PPD, Cit, IMZ and DBP were dissolved in DMSO (final concentration of DMSO in culture medium < 0.2%). Lipopolysaccharide (LPS) from *Escherichia coli* serotype 0127: B8 was obtained from Sigma and stock solution was dissolved in PBS. LPS (10 ng/mL) was used as positive control in all the experiments with THP-1 cells and DMSO was used as vehicle control for experiments with both cell lines, THP-1 dendritic cells and NCTC 2544 keratinocytes. PPD was selected for keratinocyte activation, while Cit and IMZ were used for activation of dendritic cells based on previous experiments for the consistency and robustness of results. The concentrations of DBP were first selected according to the literature (MARTINASSO *et al.*, 2006). The concentrations of the chemical sensitizers were chosen from previous experiments in the laboratory with slight changes after cell viability tests.

2.2 CELLS

For experiments using NCTC 2544 cell line (Istituto Zooprofilattico di Brescia, Italy), cells were cultured in 24 or 96-well plates (0.5 or 0.1 mL/well, respectively) at density of 2.5×10^5 cells/mL. Cells were treated in 0.5 or 0.1 mL of RPMI 1640 containing 2 mM L-glutamine, 0.1 mg/mL streptomycin, 100 IU/mL penicillin, supplemented with 10% heated-inactivated fetal calf serum (media) and cultured at 37°C in 5% CO₂.

For experiments using THP-1 cells (Istituto Zooprofilattico di Brescia, Italy), cells were used at a density of 10^6 cells/mL in RPMI 1640 containing 2mM L-

glutamine, 0.1 mg/mL streptomycin, 100 IU/mL penicillin, 50 μ M 2-mercaptoethanol, supplemented with 10% heated-inactivated fetal calf serum (media) and cultured at 37°C in 5% CO₂. For cytotoxicity, IL-8 release, or CD86 expression, 0.5×10^6 cells were seeded in 24-well plates. For IL-8 mRNA expression 3.0×10^6 cells were seeded in 14 mL round bottom tubes.

2.3 CELL VIABILITY

NCTC cells (2.5×10^5 /mL) were seeded in a 96-well plate (0.1 mL/well) and treated with PPD alone or in association with DBP, or DMSO as vehicle control. After an incubation period of 24 hours, culture media was discarded and 100 μ L/well of MTT solution 7.5 mg/mL in culture media was added. Cells were incubated for 3 hours at 37°C, media discarded and cells lysed in 100 μ L/well of a mixture of HCl 1 N: isopropanol (1:24). The absorbance of the resulting solutions was read at a wavelength of 595 nm in a microplate reader (Molecular Devices). Based on cell viability, cells were treated using the concentration of chemicals resulting in a cell viability > 80%.

For THP-1 cells, cytotoxicity was assessed by flow cytometric evaluation of propidium iodide (PI)-stained cells. Cells were seeded in 24-well plates (0.5 mL/well) and incubated for 24 hours with Cit or IMZ alone or in association with DBP, LPS as positive control, or DMSO as vehicle control. After incubation, cells were centrifuged at 1500 rpm for 5 minutes and suspended in 500 μ L PBS containing 1 μ g/mL PI. The percentage of positive cells was analyzed using a FACSCalibur flow cytometer, and data were quantified using CellQuest software (BD, Becton Dickinson). Based on cell viability, cells were treated using the concentration of chemicals resulting in cell viability > 75%.

2.4 CYTOKINE PRODUCTION

For IL-18 production, NCTC cells were incubated with PPD (30 µg/mL) alone or in association with DBP, or with DMSO as vehicle control. After incubation, culture media was discarded, monolayers gently washed once with 1 mL of PBS and cells lysed in 0.25 mL of 0.5% Triton X-100 in PBS. Plates were stored at -80°C until measurement. Intracellular IL-18 content was assessed by specific sandwich ELISA commercially available (MBL, Nagoya, Japan). Results are expressed in pg/mg of total intracellular protein content. Limit of detection is 15.6 pg/mL. The protein content of the cell lysate was determined by the BCA method.

IL-8 release was measured in THP-1 cells after 24 hours of incubation in 24-well plates (0.5 mL/well) with Cit or IMZ alone or in association with DBP, LPS as positive control, or DMSO as vehicle control. Cell-free supernatants were obtained by centrifugation at 1500 rpm for 5 minutes and stored at -20°C until measurement. IL-8 was assessed by a specific sandwich ELISA commercially available (BD, Becton Dickinson). Results are expressed as pg/mL. Limit of detection is 15.6 pg/mL.

2.5 FLOW CYTOMETRIC ANALYSIS OF CD86 EXPRESSION

CD86 expression was evaluated by flow cytometric analysis. THP-1 cells were treated with the selected chemicals and associations, LPS or DMSO as vehicle control. After 24 hours, cells were centrifuged at 1500 rpm for 5 minutes. Supernatants were collected for IL-8 assessment, and cells were stained for 30 minutes with a specific FITC-conjugated antibody against CD86 (BD, Becton Dickinson) or with isotype control antibody at 4°C following supplier's instructions. The intensity of fluorescence and the percentage of positive cells were analyzed using FACSCalibur flow cytometer, and data were quantified using CellQuest software (BD, Becton Dickinson). 10,000 viable cells were analyzed for mean fluorescence intensity (MFI) and percentage of positively labeled cells (PC). Changes in CD86 expression are reported according to Azam *et al.* (2006) as stimulation index (SI) calculated by the following equation:

$$SI = \frac{PC_t \times MFI_t}{PC_c \times MFI_c}$$

PC_c and MFI_c stand for the percentage of positive cells and mean fluorescence intensity in control cells, whereas PC_t and MFI_t stand for the percentage and mean fluorescence intensity in chemical-treated cells.

2.6 REAL TIME RT-PCR

Total RNA was isolated from THP-1 cells after 3 hours of treatment with selected chemicals and associations using commercial available kit (TriReagent from Sigma) as described by the manufacturer. For the synthesis of cDNA, 2.0 µg of total RNA was retro-transcribed using a high-capacity cDNA archive kit from Applied Biosystems (Foster City, CA, USA) following the supplier's instructions. IL-8 gene expression was evaluated by Real Time reverse transcription polymerase chain reaction (Real Time PCR). For PCR-analysis, Taq-ManTM-PCR technology was used. For each PCR reaction, 10 ng of total RNA were used. The 18S ribosomal RNA transcription was used as endogenous reference and the quantification of the transcripts was performed by the $\Delta\Delta C_T$ method.

2.7 DETERMINATION OF INTRACELLULAR REACTIVE OXYGEN SPECIES

For determination of intracellular reactive oxygen species (ROS), the fluorescent intensity of dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes, Eugene, OR, USA) oxidized product, DCF, was measured. THP-1 cells (1 mL in round bottom cytometer tubes) were loaded with DCFH-DA (2 µM) for 15 minutes and kept away from light. After fixation, cells were analyzed with FACScan flow cytometry at time 0, and then treated with DBP (500 µM/mL), Cit (15 µg/mL), Cit + DBP, LPS (10 ng/mL) as positive control, or DMSO as vehicle control. Cells were then acquired at different time points: 5, 15, 30, and 60 minutes. The fluorescence intensity of fluorophore DCF, which was formed by peroxide oxidation of the non-

fluorescent precursor, was measured (in the FL1 channel set for log scale) using FACSCalibur flow cytometer, and data were quantified using CellQuest software (BD, Becton Dickinson).

2.8 DATA ANALYSIS

All experiments were performed at least two times, with representative results shown. The data presented are expressed as mean \pm SD. Statistical significance was determined by ANOVA followed by Tukey multiple comparison test using the GraphPad Prism software (GraphPad Software, La Jolla, CA). The comparison of IMZ with negative control (DMSO) in the analysis of mRNA was performed by unpaired t test with Welch's correction. Differences were considered significant for $p \leq 0.05$.

3 RESULTS

3.1 EFFECT OF THE SELECTED CHEMICALS ON CELL VIABILITY

The chemical concentrations resulting in 80% of viability in relation to vehicle treated NCTC cells 24 hours after treatment were calculated for PPD and the association of this chemical with three concentrations of DBP. Based on these results, a concentration of 30 µg/mL of PPD and 1000 µM of DBP were selected. Two lower concentrations of DBP (500 and 250 µM) were selected to evaluate a concentration response of the adjuvant effect of this substance.

THP-1 cells were treated with increasing concentrations of the selected chemicals and associations for 24 hours. Cell viability was then assessed by flow cytometry using PI staining. According to the results, based on cell viability, cells were then treated with concentrations of chemicals resulting in more than 75% of viability, which corresponded to 15 µg/mL of Cit and 30 µg/mL of IMZ. DBP was used in the same concentrations as with NCTC.

3.2 EFFECTS OF THE SELECTED CHEMICALS ON THE RELEASE OF IL-18

NCTC cells were treated for 24 hours with PPD alone or in association with one of the three concentrations of DBP. PPD induced an increase in intracellular IL-18 content, but there was no higher increase in the groups exposed to the association between PPD and any of the concentrations of DBP. DBP alone did not induce the release of IL-18 (data not shown).

3.3 EFFECTS OF THE SELECTED CHEMICALS ON THE RELEASE OF IL-8

THP-1 cells were first treated for 24 hours with the selected concentrations of DBP. None of the concentrations of DBP were able to induce the release of IL-8 (data not shown).

Cells were then treated with Cit or IMZ, or the associations between these chemicals and the increasing concentrations of DBP. As shown in Figure 1, Cit induced the release of IL-8, and, the three concentrations of DBP in association significantly increased this release. Similar results were obtained when IMZ was associated with increasing concentrations of DBP. All concentrations of DBP were able to increase the IL-8 release induced by IMZ (FIGURE 1).

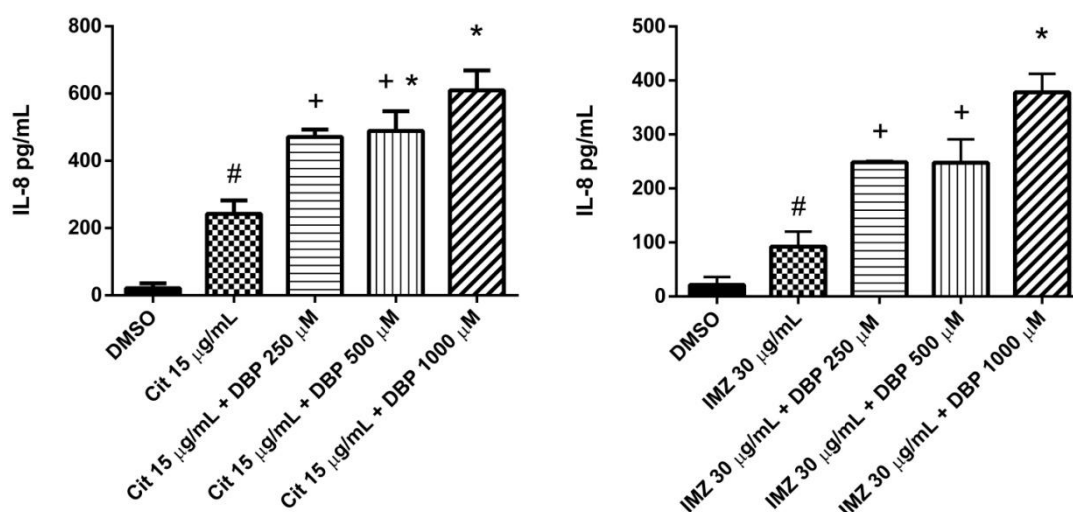


FIGURE 1. EFFECTS OF THE SELECTED CHEMICALS AND ASSOCIATIONS ON IL-8 RELEASE. THP-1 cells were treated for 24 h with Citral (Cit), Imidazolidinyl urea (IMZ) or the associations between Cit or IMZ with increasing concentrations of Dibutyl phthalate (DBP). Vehicle treated cells were included as a vehicle control (DMSO). IL-8 release was measured by ELISA in culture supernatants, results are expressed as pg/mL. Each value represents the mean \pm SD, $n=3$. Different symbols over the bars represent significant statistical difference. $p<0.05$, ANOVA followed by Tukey multiple comparison test.

3.4 CD86 EXPRESSION AFTER CITRAL, IMZ AND DBP EXPOSURE

In parallel to IL-8 release, the effect of the selected chemicals and associations on CD86 expression was investigated. Similar to the results obtained with IL-8 release, exposure to Cit showed an increase in the expression of CD86, which was significantly higher in the groups exposed to the associations between Cit with DBP (FIGURE 2). IMZ was also able to increase CD86 expression and higher expressions were observed when IMZ was associated with the concentrations of 500 and 1000 μ M of DBP, but not with 250 μ M (FIGURE 2). DBP alone did not induce the expression of CD86 in any of the concentrations (data not shown).

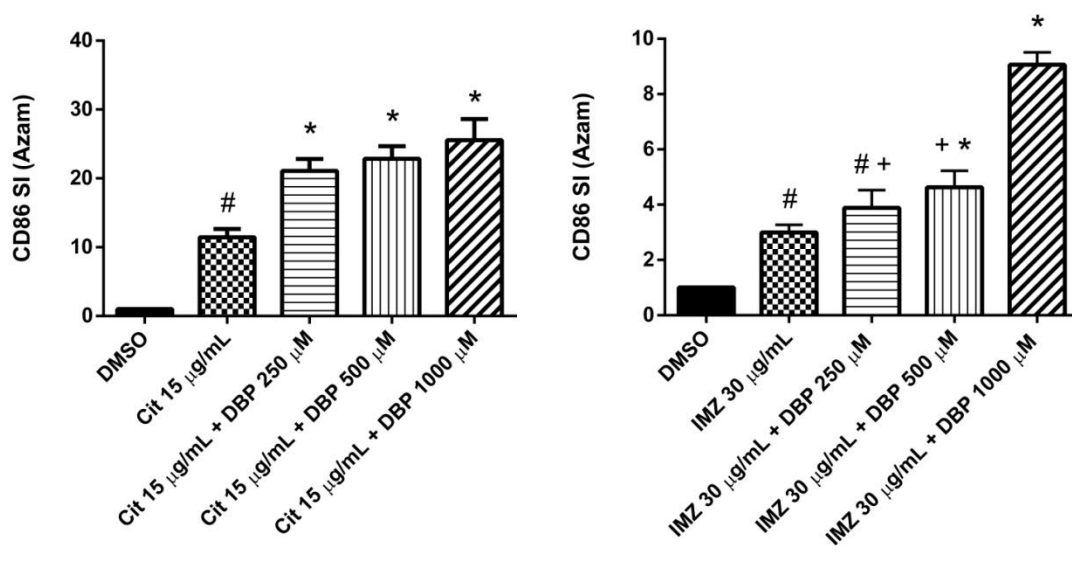


FIGURE 2. EFFECTS OF THE SELECTED CHEMICALS AND ASSOCIATIONS ON THE EXPRESSION OF CELL SURFACE MARKER CD86. THP-1 cells were treated for 24h with Citral (Cit), Imidazolidinyl urea (IMZ) or the associations between Cit or IMZ with increasing concentrations of Dibutyl phthalate (DBP). Vehicle treated cells were included as a vehicle control (DMSO). Results are expressed as stimulation index (SI). Each value represents the mean \pm SD, $n=3$. Different symbols over the bars represent significant statistical difference. $p<0.05$, ANOVA followed by Tukey multiple comparison test.

3.5 EFFECTS ON IL-8 mRNA EXPRESSION

Since IL-8 seems to be a more sensible marker on distinguishing sensitizers, we investigated the expression of the IL-8 mRNA to investigate if the adjuvant effect

of DBP in the release of IL-8 occurred before or after the transcription of IL-8 mRNA. THP-1 cells exposed to Cit showed IL-8 mRNA expression as assessed by Real Time PCR. IMZ induced a 2.7 fold increase in the IL-8 mRNA expression when compared with DMSO. When ANOVA was performed to analyze all the groups for this parameter (DMSO, IMZ, DBP and IMZ associated to DBP), we could not identify a significant difference between IMZ and DMSO because the increase in the association was too high and overshadowed this result. However, when we compared only DMSO with IMZ in a t test, the significant difference was apparent. Both Cit and IMZ, when associated with 500 μ M of DBP, presented a significant increase in mRNA expression (FIGURE 3). DBP, which tested negative for the release of IL-8 after 24 hours of treatment, was able to induce the mRNA expression after 3h of incubation (FIGURE 3).

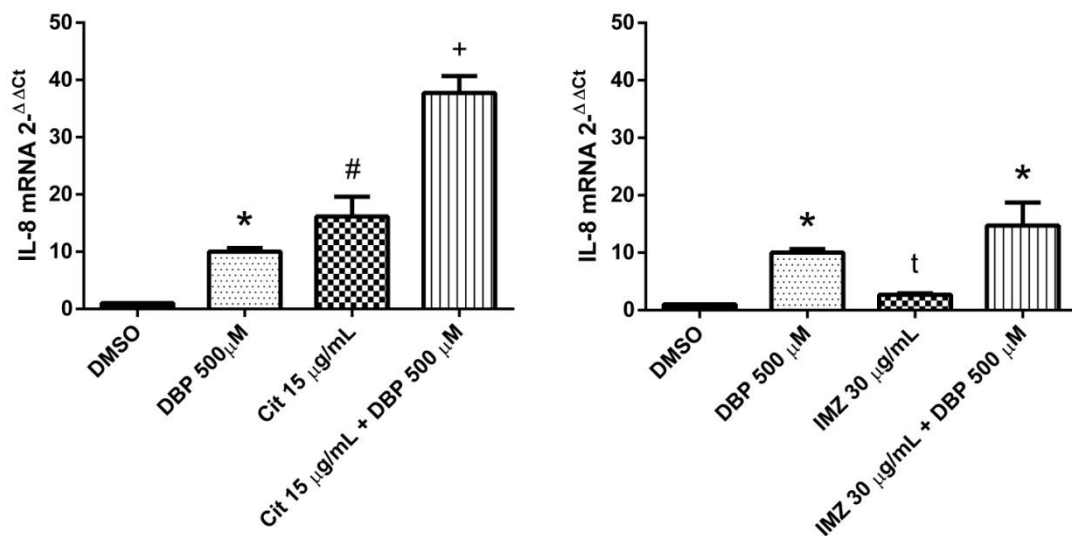


FIGURE 3. EFFECTS OF THE SELECTED CHEMICALS AND ASSOCIATIONS ON IL-8 mRNA EXPRESSION. THP-1 cells were treated for 3 h with Citral (Cit), Imidazolidinyl urea (IMZ), Dibutyl phthalate (DBP) or the associations between Cit or IMZ with DBP. Vehicle treated cells were included as a vehicle control (DMSO). IL-8 mRNA expression was evaluated by Real Time-PCR as described in the Material and Methods section. Results are expressed as $2^{-\Delta\Delta C_T}$. Each value represents the mean \pm SD, n=3 independent experiments. Different symbols over the bars represent significant statistical difference. p<0.05, ANOVA followed by Tukey multiple comparison test. (t) Significant difference when compared with DMSO. p<0.05, Unpaired t test with Welch's correction.

3.6 EFFECTS ON INTRACELLULAR REACTIVE OXYGEN SPECIES (ROS)

Figure 4 shows the ability of the selected chemicals to generate reactive oxygen species assessed by the DCFH-DA assay in THP-1 cells. The exposure to Cit increased DCFH-detected ROS levels at 5 minutes of treatment, which was maintained until the end of the evaluation (60 min). This increase was enhanced when Cit was associated with 500 μ M of DBP in all evaluated time points. DBP alone also induced a slight increase in ROS, and this was more evident at 5 minutes of treatment.

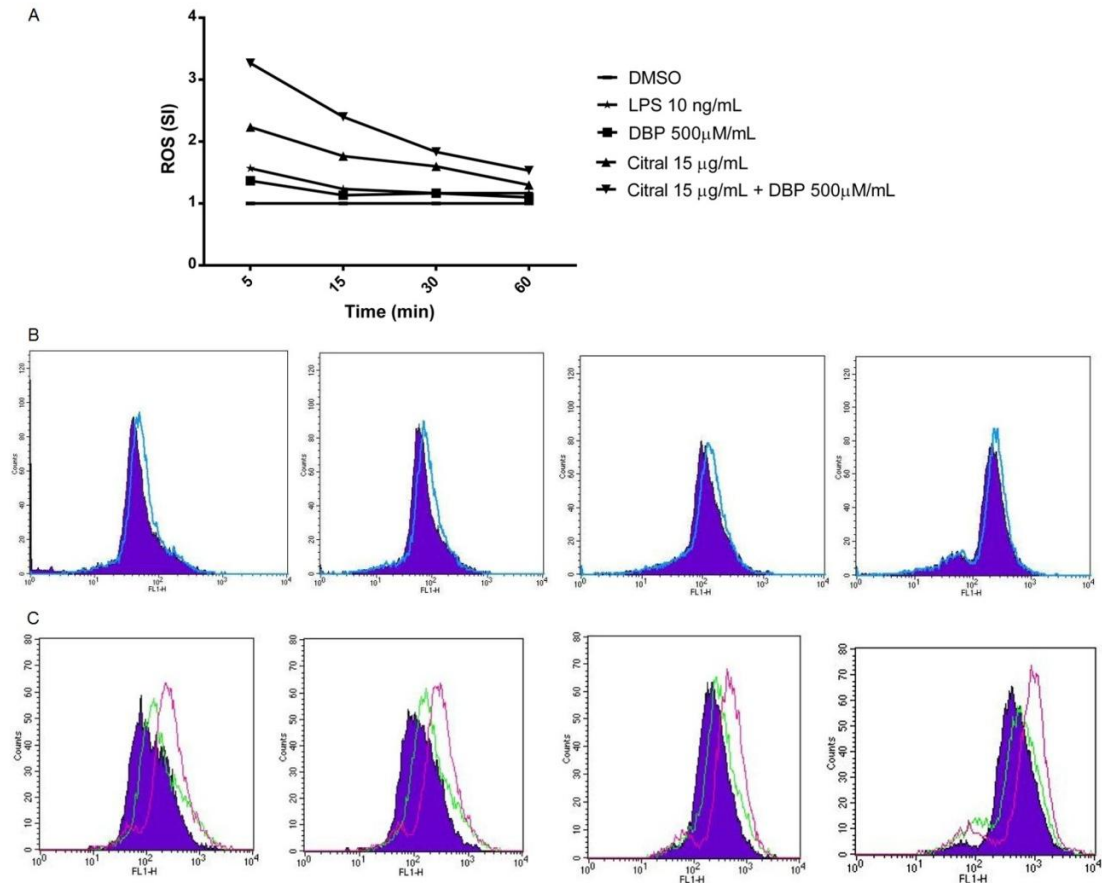


FIGURE 4. EFFECTS OF THE SELECTED CHEMICALS AND ASSOCIATIONS ON INTRACELLULAR REACTIVE OXYGEN SPECIES. THP-1 cells were loaded with DCFH-DA and then treated with Dibutyl phthalate (DBP, 500 μ M), Citral (Cit, 15 μ g/mL), Cit + DBP, LPS (10 ng/mL) as positive control, or DMSO as vehicle control. Cells were then acquired at different time points: 5, 15, 30, and 60 minutes. The fluorescence intensity of fluorophore DCF was measured by flow cytometry as described in the Material and Methods section. Results are expressed as stimulation index (SI). (A) Each value represents the mean, $n=3$ independent experiments. (B) THP-1 cells were treated with DBP 500 μ M (blue) for 5, 15, 30 and 60 min. The purple histogram represents control cells. (C) THP-1 cells were treated with Cit 15 μ g/mL (green) or Cit associated with DBP 500 μ M (pink) for 5, 15, 30 and 60 min. The purple histogram represents control cells.

4 DISCUSSION

It has been postulated that phthalate esters may be associated with facilitation in the development of allergic diseases such as asthma and atopic dermatitis (BORNEHAG *et al.*, 2004; JAAKKOLA; KNIGHT, 2004). Additionally, certain phthalates have been shown to enhance the response to allergens in animal models of allergic contact dermatitis (IMAI *et al.*, 2006; LI *et al.*, 2014). The underlying mechanisms, however, have not been fully elucidated. The present *in vitro* study investigated the effects of the plasticizer DBP on chemical allergen-induced cell activation. In particular, NCTC 2544 and IL-18 production were used as surrogate of contact allergen-induced keratinocyte activation, while the upregulation of CD86 and IL-8 production in THP-1 cells were used as markers of dendritic cell (DC) activation. Results obtained suggest an adjuvant effect of DBP in DC but not in epidermal keratinocytes, as demonstrated by the lack of co-stimulation in PPD-induced IL-18 production.

Since humans are commonly exposed to phthalates, it is important to elucidate potential immunotoxic effects of the same. With the widespread use of DBP, its potential toxicity is getting increasing attention. It is known that phthalate esters lack the ability to bind to proteins and form a hapten-protein complex, which is required for the stimulation of allergic sensitization (KIMBER; DEARMAN, 2010). According to epidemiological data, phthalate esters do not have the potential to cause dermal irritation or skin sensitization (MEDEIROS; DEVLIN; KELLER, 1999; GEIER *et al.*, 2004). However, even though evidence supports a lack of sensitizing potential, it has been suggested that certain phthalates may act as adjuvants by enhancing immune responses.

The NCTC 2544 IL-18 assay is a useful *in vitro* tool that helps identify contact sensitizers and discriminate them from irritants and respiratory allergens (CORSINI *et al.*, 2009; GALBIATI *et al.*, 2011; CORSINI *et al.*, 2013a). In this study, as expected, the contact allergen PPD induced a statistically significant increase in the production of IL-18, while DBP alone failed to induce it, supporting the evidence that phthalates lack the potential to cause sensitization. The association with DBP failed, however, to further increase IL-18 production, suggesting that DBP does not act as an adjuvant in keratinocytes.

Mitjans *et al.* (2008) demonstrated that chemical allergens induce a concentration-related release of interleukin-8 as well as the expression of the surface marker CD86 in the human promyelocytic cell line THP-1, proposing this as an *in vitro* assay for the screening of potential chemical allergens. In this work, the CD86 upregulation as well as IL-8 release were investigated after exposure of THP-1 cells to two contact allergens, citral and imidazolidinyl urea, and their associations with DBP. According to the local lymph node assay (LLNA), citral is classified as moderate sensitizer with an EC3 value of 9.3%, while imidazolidinyl urea is classified as a weak sensitizer with an EC3 value of 24%. Both allergens induced a statistically significant upregulation in the expression of CD86. DBP alone did not induce this expression, further supporting the lack of sensitizing potential. In accordance with our data, Ito *et al.* (2012) showed that di (2-ethylhexyl) phthalate (DEHP) did not induce the expression of dendritic cells differentiation and maturation markers, including CD86, in mouse peripheral blood mononuclear-derived dendritic cells. On the contrary, Koike *et al.* (2009; 2010) using bone marrow-derived dendritic cells obtained from atopic prone NC/Nga mice, found that DEHP as well as diisononyl phthalate (DINP) enhanced the expression of CD86 and other costimulatory molecules. Different culture conditions, cells or experimental design could explain these differences.

The exposure of THP-1 cells to Cit and IMZ revealed a statistically significant release of IL-8 compared to vehicle treated cells. Again, DBP alone failed to induce the release of IL-8. Data on phthalate-induced release of cytokines *in vitro* is conflicting. Although it has been reported that some phthalate monoesters, including the DBP metabolite monobutyl phthalate (MPB), do not induce the release of cytokines after exposure in THP-1 cells (GLUE *et al.*, 2002), others have shown that phthalate monoesters lead to the release of IL-8 in the epithelial cell line A549 (JEPSEN; ABILDTRUP; LARSEN, 2004). In the study by Jepsen *et al.* (2004), high concentrations of MBP induced only a slight increase in IL-8 release, and more significant increases were obtained with the exposure to monoesters with more than eight carbon atoms in their alkyl side chain. According to their results, these authors suggest that phthalate monoesters with fewer than eight carbon atoms in their alkyl side chain (like MBP) are weak cytokine inducers, whereas monoesters with eight or more carbon atoms in the alkyl side chain are more potent cytokine inducers.

When THP-1 cells were exposed to either Cit or IMZ associated with DBP, cells exhibited higher expression of CD86 and release of IL-8, when compared to the exposure to the allergen alone. This indicates an adjuvant effect of DBP on THP-1 cells. Even though several studies have been performed to evaluate the effects of phthalates in cell function, little can be found in the literature regarding the adjuvant effect of these substances *in vitro*. Nevertheless, a great amount of studies have been performed about the adjuvant effect of phthalates *in vivo*. Several authors have showed that different phthalates can act as adjuvants in contact hypersensitivity and airway inflammation, elevating the expression of surface markers and related cytokines after exposure to associations between allergens and phthalates by different protocols (IMAI *et al.*, 2006; KOIKE *et al.*, 2010; MATSUDA *et al.*, 2010; LI *et al.*, 2014). Corroborating our results, Tanaka *et al.* (2012) demonstrated that DEHP exacerbated ovalbumin-related peritoneal inflammation characterized by enhanced infiltration of macrophages and neutrophils and amplified production/release of cytokines such as keratinocyte-derived chemoattractant (KC) in the peritoneal cavity. KC is a murine homolog of IL-8 (HOL; WILHELMSSEN; HARALDSEN, 2010). Furthermore, Takano *et al.* (2006) showed that exposure to DEHP in the presence of allergen increased the expression of MIP-1 α compared to vehicle combined with allergen only. MIP-1 α functions as chemotactic for neutrophils similarly to IL-8 (HAMMOND *et al.*, 1995; MENTEN; WUYTS; VAN DAMME, 2002).

According to Galbiati *et al.* (2012) the expression of IL-8 mRNA at 3 hours is important since some chemical allergens fail to induce the release of IL-8 after 24 hours of exposure, but all chemical sensitizers tested induced IL-8 mRNA at 3 hours. Both Cit and IMZ induced the expression of IL-8 mRNA, and this expression was significantly higher when associated with 500 μ M of DBP. As IMZ is a weak sensitizer, its induction of the expression of IL-8 mRNA was lower compared to the induction following Cit exposure. IMZ induced a 2.7 fold increase in IL-8 mRNA, still significantly different from the control group (Welch's t test). Interestingly, DBP, which failed to induce the expression of the surface marker CD86 or the release of IL-8, induced a significant expression of IL-8 mRNA. As mentioned before, data about the release of pro-inflammatory cytokines, including IL-8, induced by phthalates is conflicting. While some authors found that certain monophthalates can induce this release at high concentrations, others report no release after monophthalates exposure (GLUE *et al.*, 2002; JEPSEN *et al.*, 2004). Overall, little can be found in

literature relating DBP exposure and increase in pro-inflammatory cytokines mRNA expression. The control of translation and mRNA degradation is an important part of the regulation of gene expression. MicroRNAs (miRNAs) are a class of small RNA molecules that modulate gene expression by interacting with the messenger RNA of the respective target gene (SCHNEIDER, 2012). miRNAs can silence mRNAs either by triggering endonuclease cleavage, by promoting translation repression, or by accelerating mRNA decapping (VALENCIA-SANCHEZ *et al.*, 2006). It's been shown that certain miRNAs are upregulated in allergic contact dermatitis (VENNEGAARD *et al.*, 2012) and other skin inflammatory diseases (SONKOLY *et al.*, 2010; ZIBERT *et al.*, 2010), although still scarce information can be found about the function of miRNAs in skin sensitization. Since the silencing of mRNAs by miRNAs occurs through a very specific interaction between the miRNA and its targeted mRNA (VALENCIA-SANCHEZ *et al.*, 2006), this could be a possible explanation to why only the IL-8 mRNA upregulated by DBP was not translated into protein. However, this is something that needs further investigation.

Although previous *in vivo* studies have shown that certain phthalates aggravate allergic response and/or diseases (even though in doses considerably higher than those relevant for human exposure), the underlying cellular and molecular mechanisms remain unclear. Evidence indicates that allergic and inflammatory skin diseases could be mediated by oxidative stress (OKAYAMA, 2005; BYAMBA *et al.*, 2010). Mizuashi *et al.* (2005) hypothesized that sensitizers induce oxidative stress in dendritic cells, which subsequently stimulates p38 MAPK. Their results demonstrated that sensitizers reduced the oxidized (GSSG) versus reduced (GSH) form of cellular glutathione (GSH/GSSG ratio) in THP-1 cells and phosphorylated p38 MAPK. According to Mitjans *et al.* (2008), the activation of the p38 MAPK by sensitizers plays a crucial role in the activation of IL-8 release. In this study, together with increase in IL-8 release, we showed a slight increase of ROS generation induced by DBP alone, but, more importantly, a significant increase in the oxidative stress induced by DBP when in association with Cit. Others have demonstrated that some phthalates can induce oxidative stress. Tetz *et al.* (2013) reported an increase in ROS production induced by MEHP in human placental cells. DEHP also increased the generation of ROS in testis inducing apoptosis of spermatocytes (KASAHARA *et al.*, 2002). Moreover, You *et al.* (2014) showed significantly higher levels of ROS in ovalbumin sensitized mice that were co-treated

with DEHP when compared to the levels of ROS of the sensitized-only group. These authors suggest that oxidative stress plays a critical role in the adjuvant effect of DEHP.

In the present study we demonstrated a positive adjuvant effect of DBP when associated with citral and imidazolidinyl urea as indicated by the increased expression of CD86, IL-8 mRNA and IL-8 release. We also suggest that the increased generation of ROS could be the trigger that leads to these effects. On the contrary, we found no adjuvant effect on keratinocytes. Taken together, our results support that the adjuvant effect of DBP relies on dendritic cells. Previously published data corroborate our results. Koike *et al.* (2009) and Ito *et al.* (2012), based on their *in vivo* and *in vitro* studies, suggest that DEHP accelerates murine dermatitis through the inappropriate activation of dendritic cells. Koike *et al.* (2010) proposed that DINP may activate dendritic cells in inflammatory sites through the enhancement of thymic stromal lymphopoietin (TSLP) production. The production of TSLP has also been shown to be induced by DBP (LARSON *et al.*, 2010). Imai *et al.* (2006) suggested that DBP and DPP facilitate the trafficking of Langerhans cells and/or myeloid dendritic cells from skin sites in contact hypersensitivity induced by fluorescein isothiocyanate.

In an immunological context an adjuvant is a material that is able to enhance immune responsiveness without itself necessarily providing any specific antigenic stimulus (KIMBER; DEARMAN, 2010). From the results on CD86 expression and IL-8 release, where DBP alone had no effects, an enhanced upregulation of both markers was observed when DBP was associated to Cit or IMZ, suggesting that DBP acts as an adjuvant by immunological definition. Since evidence that phthalates can act as sensitizers themselves is scarce and as other authors have suggested in different animal models of dermatitis that phthalates act as adjuvants, we believe that the effects found in our work are consistent with an adjuvant effect. Interestingly, this effect seems to be specific for immune cells as no additive effects were observed in chemical allergen-induced IL-18 production in keratinocytes in the presence of DBP.

In conclusion, we propose that DBP presents *in vitro* an adjuvant effect when associated with sensitizers. However, the effects of DBP *in vivo* remain incompletely understood, thus, future studies are necessary to elucidate the precise mechanism of adjuvant effect of DBP not only *in vitro* but also *in vivo*.

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5.2.1 Material complementar

5.2.1.1 Highlights

Adjuvant effect of dibutyl phthalate in allergen induced cell activation is reported.

The adjuvant effect was observed in dendritic cells but not in keratinocytes.

Oxidative stress plays a role in the molecular mechanism of this adjuvant effect.

5.2.1.2 Graphic abstract

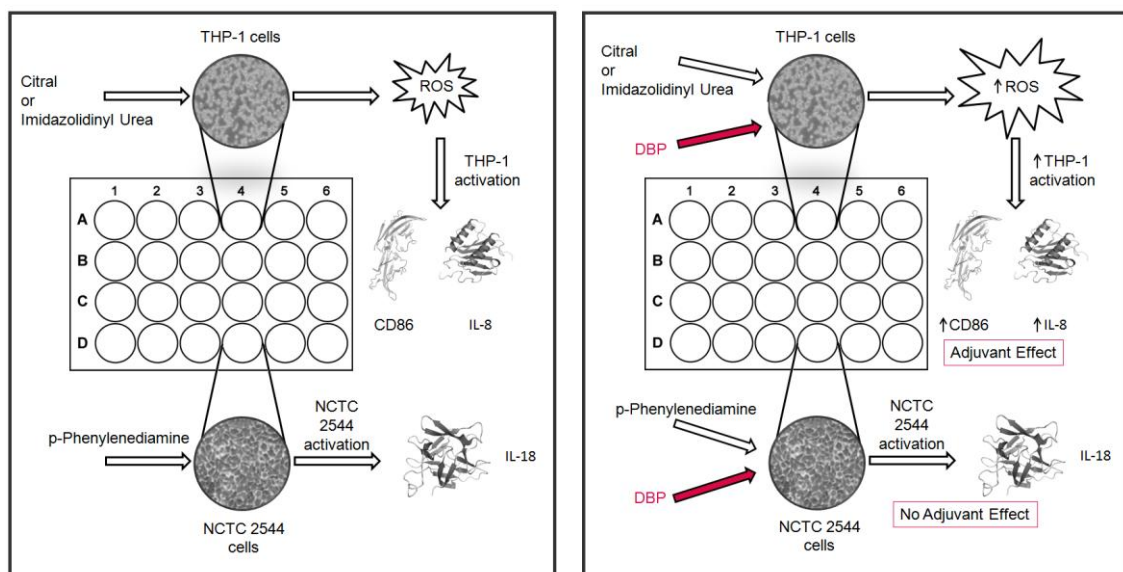


FIGURA 06. GRAPHIC ABSTRACT.

5.2.1.3 Figuras complementares

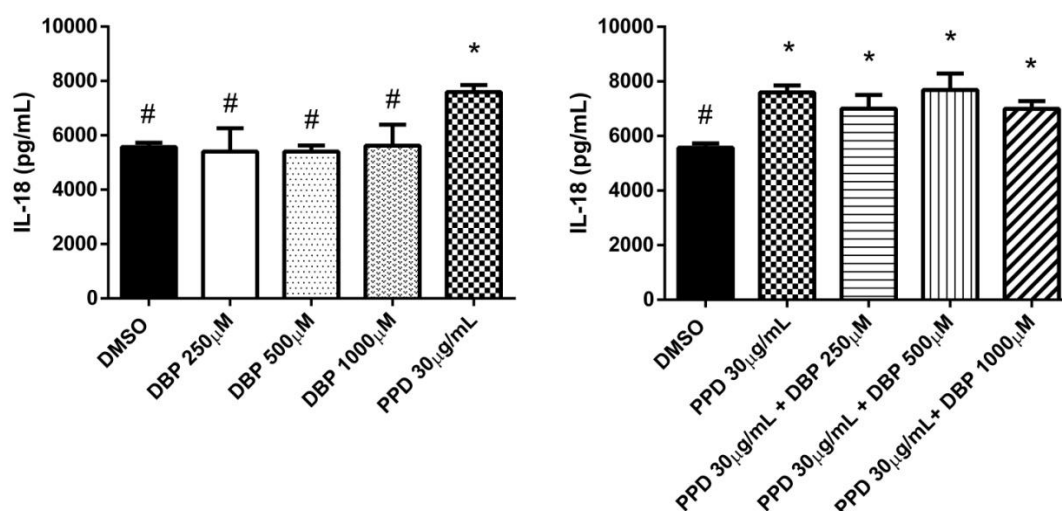


FIGURA 07. EFEITOS DO DIBUTIL FTALATO, ISOLADO OU ASSOCIADO À p-FENILENODIAMINA, NO CONTEÚDO DE IL-18 INTRACELULAR. Células NCTC 2544 foram incubadas por 24h com concentrações crescentes de DBP ou PPD associado a concentrações crescentes de DBP. O conteúdo intracelular de IL-18 foi avaliado por ELISA. Resultados são expressos em pg/mg de conteúdo total de proteína intracelular. O conteúdo total de proteína no lisado celular foi determinado pelo método BCA. Cada valor representa a média \pm desvio padrão, $n=3$. Símbolos diferentes sobre as barras representam diferença estatística significativa. $p<0.05$, ANOVA seguido de Tukey.

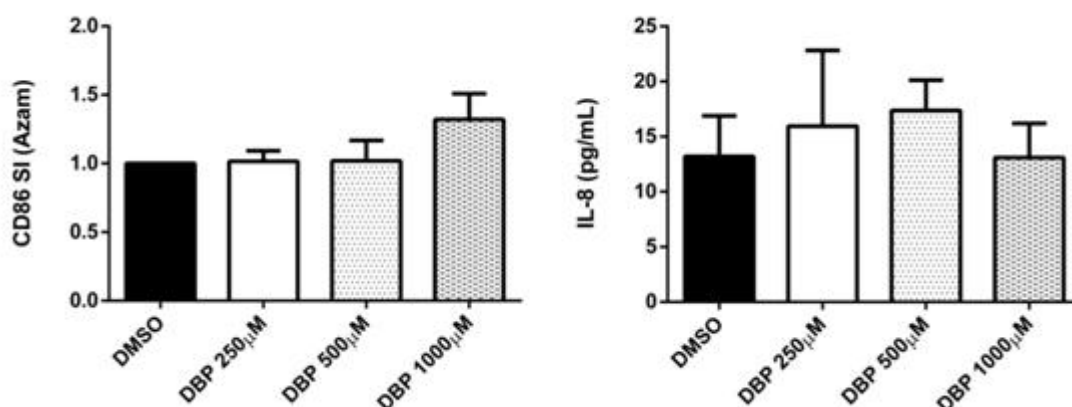


FIGURA 08. EFEITOS DO DIBUTIL FTALATO NA EXPRESSÃO DO MARCADOR DE SUPERFÍCIE CELULAR CD86 E NA LIBERAÇÃO DE IL-8. Células THP-1 foram tratadas por 24h com concentrações crescentes de Dibutil Ftlatato (DBP). Células tratadas com veículo foram incluídas como um controle negativo (DMSO). Resultados para expressão de CD86 são apresentados como índice de estimulação (SI). Cada valor representa a média \pm desvio padrão, $n=3$. Símbolos diferentes sobre as barras representam diferença estatística significativa. $p<0.05$, ANOVA seguido de Tukey.

5.3 ARTIGO 2

The plasticizer dibutyl phthalate aggravates contact hypersensitivity induced by oxazolone in BALB/c mice.

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ABSTRACT

Phthalate esters have been reported to participate in the development of allergic diseases acting as adjuvants. Contact hypersensitivity (CHS) is an allergic reaction resulting from repeated contact with chemicals called haptens, consisted in two phases: sensitization and elicitation. The present study aimed to investigate the effects of dibutyl phthalate (DBP) in the elicitation phase of CHS induced by oxazolone in an animal model of ear edema. For the experiments, adult male BALB/c mice were sensitized (in shaved abdomen) and challenged (elicitation; in the right ear) with oxazolone or vehicle (acetone) topically, and subsequently treated 6 times (with 12 hour intervals) with DBP at three doses (0.04, 0.4 or 4 mg/ear) or vehicle (acetone) in the same ear (topically). Ear thickness was measured before elicitation (baseline) and 24, 48 and 72 hours after elicitation to calculate ear edema. After the last measure, animals were euthanized and 6 mm circles of ear tissue were collected to evaluate the activity of the enzymes N-acetyl- β -d-glucosaminidase (NAG) and myeloperoxidase (MPO) (markers of leukocyte infiltration), histological analysis (infiltrated cell count, dermal and epidermal thickness) and lipid hydroperoxide (LOOH) and reduced glutathione (GSH) levels. We also investigated the adjuvant effect of DBP in oxazolone-induced THP-1 activation (an *in vitro* assay used as surrogate for dendritic cell activation). For that, THP-1 cells were exposed to oxazolone alone or associated with DBP and the expression of cluster of differentiation 86 (CD86) and interleukin 8 (IL-8) mRNA were evaluated. As expected, animals sensitized and challenged with oxazolone presented increase in ear edema, dermal and epidermal thickness, number of infiltrated cells and MPO and NAG activity, when compared with the negative control group. DBP 0.4 and 4 mg/ear increased all the parameters induced by oxazolone, suggesting an enhancement of the inflammatory response. Oxazolone also increased the levels of LOOH and reduced GSH, and these effects were partially or completely reversed by DBP 0.4 and 0.04 mg/ear, respectively. These results indicate that DBP aggravates the contact hypersensitivity induced by oxazolone. DBP also enhanced the expression of CD86 induced by oxazolone in THP-1 cells, suggesting that the enhanced activation of dendritic cells could be part of the mechanism of DBP's adjuvant effect. Our results indicate that DBP can act as adjuvant in the elicitation phase of oxazolone-induced CHS, and support data that suggest an adjuvant effect of phthalate esters in allergic diseases in certain doses.

Key words: dibutyl phthalate, oxazolone, contact hypersensitivity, allergic contact dermatitis, adjuvant effect.

1 INTRODUCTION

Phthalate esters are plasticizers highly used in a variety of manufactured products, and, due to their wide distribution in the environment, they are considered agents with high potential for human exposure (KOO; LEE, 2004; ANDERSON *et al.*, 2001; SILVA *et al.*, 2004; LYCHE *et al.*, 2009). Dibutyl phthalate (DBP) can be found in several products, such as plastics for food packing, glues, paints, solvents, perfumes, moisturizers, hairsprays, nail polishes, insect repellents and children's products (IPCS, 1997; SATHYANARAYANA *et al.*, 2008).

DBP, as well as other phthalates, have attracted great attention of the scientific community due to the great possibility of human exposure, as well as their possible endocrine disrupting effects that might lead to alterations in the development of male reproductive tract *in utero* (FISHER *et al.*, 2003; HEUDORF; MERSCH-SUNDERMANN; ANGERER, 2007; MARTINO-ANDRADE *et al.*, 2009). However, besides the effects on endocrine and reproductive systems, epidemiological and experimental data indicate other possible adverse effects due to the exposure to these substances. It is believed that certain phthalates can exacerbate the potency of allergens, and, so, play a role in the development and/or aggravation of allergic diseases (CASILLAS *et al.*, 1999). Experimental studies show that phthalate esters, such as DBP, are well absorbed by the skin, but irritation and sensitization tests indicate no evidence of skin inflammatory responses by these substances (ELSISI; CARTER; SIPES, 1989; DOAN; BRONAUGH; YOURICK, 2010; BEYDON; PAYAN; GRANDCLAUDE, 2010; CPSC, 2010; PAN *et al.*, 2014). Therefore, in some countries, like in Brazil, these substances are still allowed in cosmetic formulations. However, most available dermal studies do not include the possible adjuvant effect of phthalates in hypersensitivity models. Even though phthalate esters are not capable of inducing hypersensitivity themselves, it has been reported that they are capable of exacerbating the inflammatory response in contact hypersensitivity in animal models (CHOWDHURY; STATHAM, 2002; IMAI *et al.*, 2006; MATSUDA *et al.*, 2010; LI *et al.*, 2014). These studies indicate that the dermal toxicity of phthalate esters needs better evaluation and attention.

Allergic contact dermatitis (ACD) or contact hypersensitivity (CHS) is a clinical manifestation in response to repeated or prolonged contact to a chemical

substance called hapten (HENNINO *et al.*, 2005; KARLBERG *et al.*, 2008), and it is considered one of the most important manifestations of immunotoxicity in humans (KIMBER, 2002). Allergic contact dermatitis is a delayed type hypersensitivity reaction mediated by antigen specific T lymphocytes (KARLBERG *et al.*, 2008), and can be characterized in two phases: sensitization (first contact with the hapten, antigen presenting to the lymph node and activation of specific T lymphocytes) and elicitation (subsequent contacts with hapten, recognition by specific T lymphocytes and development of inflammatory response) (GRABBE; SCHWARZ, 1998; KIMBER, 2002; HENNINO *et al.*, 2005; KARLBERG *et al.*, 2008).

Several cell types are involved in the contact hypersensitivity response. Dendritic cells are recognized as important antigen presenting cells in adaptive immunity because of their capacity to stimulate naive lymphocytes (BANCHEREAU *et al.*, 2000). Mitjans *et al.* (2008) reported in the human promyelocytic cell line THP-1 (used as a surrogate of dendritic cells), a selective production of the chemokine interleukin 8 (IL-8) by contact allergens in parallel to the surface marker cluster of differentiation 86 (CD86) upregulation, and, proposed this as a possible *in vitro* sensitization test. IL-8 is a potent attractant for neutrophils and different subtypes of T cells (BARKER *et al.*, 1991). We have previously demonstrated that DBP can potentiate citral and imidazolidinyl urea-induced THP-1 activation, suggesting that part of the mechanism of DBP's adjuvant effect could be through enhanced activation of dendritic cells (LOURENÇO *et al.*, 2015).

It has been reported that oxidative stress can play a central role in chemical-induced contact hypersensitivity and inflammatory diseases (OKAYAMA, 2005; BYAMBA *et al.*, 2010; CORSINI *et al.*, 2013). Besides, evidence that phthalates are associated to oxidative stress has been shown by several authors. Seo *et al.* (2004) and Tetz *et al.* (2013) have demonstrated increased generation of reactive oxygen species (ROS) induced by phthalates in rats and in a human placental cell line, respectively, and others have shown associations between urinary concentrations of phthalate monoester metabolites and increased markers of inflammation and oxidative stress in humans (HAUSER *et al.*, 2007; FERGUSON; LOCH-CARUSO; MEEKER, 2011).

Prior animal studies on hypersensitivity contact have used high phthalate doses and failed to comprehensively characterize the inflammatory process involved in the adjuvant effects of phthalates (TAKANO *et al.*, 2006; MATSUDA *et al.*, 2010).

With this study, we aimed to investigate the possible adjuvant effects of lower DBP doses (KOO; LEE, 2004; INTERNATIONAL JOURNAL OF TOXICOLOGY, 2005) in the elicitation phase of contact hypersensitivity induced by oxazolone in an animal model of ear edema. We also investigated the possible role of oxidative stress in this adjuvant effect, besides evaluating the effects of DBP in oxazolone-induced THP-1 activation.

2 MATERIAL AND METHODS

2.1 ANIMALS

Adult male BALB/c mice obtained from Universidade de Campinas (Unicamp – Campinas, SP, Brazil) were used for the experiments. The animals were kept in the animal facilities of the Reproductive Toxicology Laboratory (Departamento de Farmacologia, Universidade Federal do Paraná, Curitiba, PR, Brazil) under a 12 h light/dark cycle and controlled temperature ($22 \pm 2^\circ\text{C}$). Standard pellet food and tap water were provided *ad libitum*. The experimental protocol was approved by the Committee on Animal Research and Ethics of the Universidade Federal do Paraná (Curitiba, Brazil) under the number 633.

2.2 CHEMICALS AND DOSE SELECTION

Dibutyl phthalate (DBP, CAS number 84-74-2, purity 99%) and Oxazolone (4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one, CAS number 15646-46-5) were obtained from Sigma-Aldrich (St. Louis, MO, USA). DBP and oxazolone were diluted in acetone (vehicle) for *in vivo* experiments, and in DMSO for *in vitro* experiments (final concentration of DMSO in culture medium $< 0.2\%$).

DBP *in vivo* doses were based on DBP exposure estimations from use of personal care products, in particular perfumes. According to Koo and Lee (2004), the average concentration of DBP found in a variety of perfumes is 0.44 mg/mL, and the average use of perfume by the general population, estimated through an applied questionnaire, is 1mL/day. Therefore, the amount of DBP applied to the skin in individuals that use determined kinds of perfumes can be approximately 0.4 mg/day, which corresponds to 0.0067 mg/kg/day for a 60 kg individual or 0.25 mg/m²/day using the conversion factor recommended by the FDA (FDA, 2005). These estimations are in agreement with the data published by the International Journal of Toxicology (2005) in an annual review on safety of ingredients from cosmetic

products, indicating that the use of perfumes may result in skin applications of up to 0.47 mg DBP/day. Based on these data we tested doses of 0.04, 0.4 and 4 mg/day in BALB/c mice. The lowest dose (0.04 mg/day) corresponds to 2 mg/kg/day in a 0.02 kg mouse or 6 mg/m²/day using the conversion factor recommended for mice by the FDA publication (FDA, 2005), i.e., 24 times higher than the estimated human exposure described above (0.25 mg/m²/day). For *in vitro* experiments, concentrations of DBP (250, 500 and 1000 µM) were selected according to the literature (MARTINASSO *et al.*, 2006).

Among sensitizing substances, oxazolone was selected for being frequently used to promote the phases of sensitization and elicitation of contact hypersensitivity. Oxazolone is considered a strong hapten, with potent pro-inflammatory properties (ZHANG; TINKLE, 2000).

2.3 EVALUATION OF EAR EDEMA

Mice were sensitized with 30 µL of oxazolone (0.25% w/v in acetone) or vehicle on day 0 on shaven abdomen. After five days, baseline ear thickness was measured with an electronic digital outside micrometer (Digimess, Argentina). Animals were then challenged on the right ear with 15 µL of oxazolone (0.5% w/v in acetone) or vehicle (elicitation). After elicitation, the right ear of the animals was treated with 20 µL of vehicle or one of the three doses of DBP (0.04, 0.4 or 4 mg/ear). The treatment with vehicle or DBP was performed 5 other times in 12 hours intervals, totalizing 6 treatments by the end of 72 hours. To certify that DBP is not a sensitizing chemical in this animal model, a group of animals was sensitized and challenged with DBP (0.4 mg) and then treated with vehicle (DBP challenge group). Ear thickness was measured 24, 48 and 72 hours after elicitation (RECIO *et al.*, 2000). Ear edema was expressed as the increase of ear thickness related to the baseline measurement in micrometers (µm).

The animals were randomly divided in groups with 5 to 7 animals each (n= 5 to 7 mice/group). The treatments were performed as described in Figure 1.

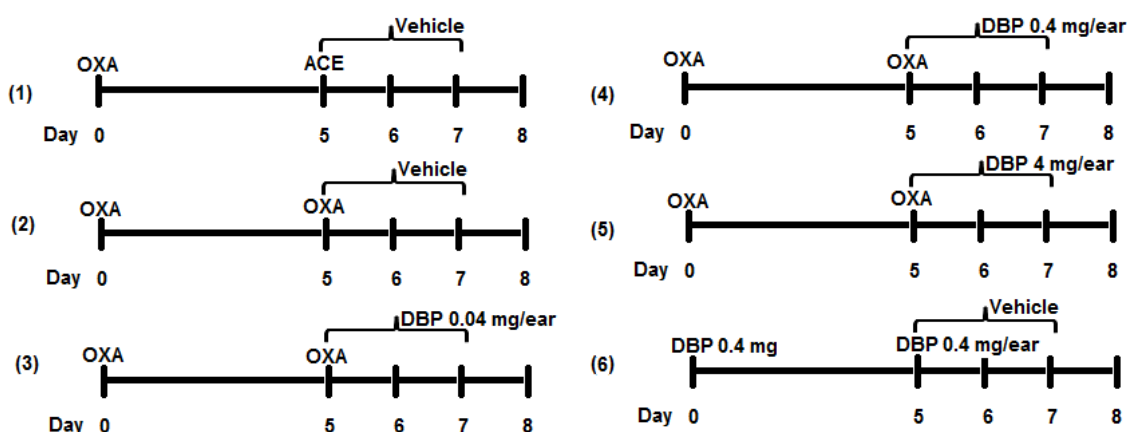


FIGURE 1. EXPERIMENTAL DESIGN. Male BALB/c mice were treated with 30 μ L of Oxazolone (OXA, 0.25% w/v) or Dibutyl phthalate (DBP, 0.4 mg) on their shaven abdomens on day 0 (Sensitization). On day 5, baseline ear thickness was measured followed by challenge with 15 μ L of vehicle (ACE, acetone), Oxazolone (0.5%) or DBP (0.4 mg) on the right ear. After challenge, the animals received 6 treatments on the right ear with DBP (0.04, 0.4 or 4 mg/ear) or vehicle. Treatments were performed with 12 hour intervals. Ear thickness was measured on days 6, 7 and 8 (24, 48 and 72 hours after challenge). On day 8, the animals were euthanized and 6 mm circles of ear tissue were collected for future analysis. $n = 5$ to 7 mice in each group. (1) Negative control group, (2) Positive control group for contact hypersensitivity, (3) Treated group with DBP 0.04 mg/ear, (4) Treated group with DBP 0.4 mg/ear, (5) Treated group with DBP 4 mg/ear, (6) DBP challenge group.

After ear edema evaluation the animals were euthanized by cervical dislocation and 6 mm circles of ear tissue were collected for posterior histological evaluation, enzyme activity assays (N-acetyl- β -D-glucosaminidase - NAG and myeloperoxidase - MPO), and reduced glutathione (GSH) and hydroperoxide (LOOH) content. The ears of naïve animals (that did not receive any treatment) were collected for evaluation of MPO and NAG activity and GSH and LOOH content.

2.4 HISTOLOGICAL ANALYSIS

Ear tissue samples were fixed in ALFAC (10% formaldehyde, 85% ethanol 80%, 5% glacial acetic acid) solution. The ears were later embedded in paraffin, cut at 5 μ m sections and stained with hematoxylin–eosin.

The images for analysis were obtained through a motorized microscope Axio Imager Z2 (Carl Zeiss, Jena, Germany), equipped with automated scanner VSlide (Metasystems, Altlussheim, Germany). The scanned images were analyzed with ImageJ software (NIH, USA). Epidermal thickness (considered as the distance from

the basal layer to the stratum granulosum) and dermal thickness (considered as the distance from the basal layer of the epidermis to the auricular cartilage) were measured, and the presence of inflammatory cells was quantified. Data are expressed as the mean value of three representative areas of three histological sections of each animal. Epidermal thickness measure and infiltrated cells count were performed in 200x magnification sections, while dermal thickness was measured in 100x magnification sections.

2.5 TISSUE MYELOPEROXIDASE (MPO) ASSAY

MPO is an enzyme used as a marker for the influx of polymorphonuclear leukocytes into inflamed tissues. MPO activity was evaluated according to the method proposed by Bradley *et al.* (1982) and modified by De Young *et al.* (1989). Each ear sample (6 mm circles) was placed in 0.75 mL of 80 mM sodium phosphate buffer (PBS, pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB). Next, the sample was homogenized (45 s at 0 °C) in a motor-driven homogenizer. The homogenate was decanted into a microfuge tube, and 0.75 mL of HTAB in PBS was added to the tube. The 1.5 mL mixture was centrifuged at $11,200 \times g$ at 4 °C for 20 min. The supernatant samples (triplicates of 30 μ L) were added to 96-well plates. For the assay, 200 μ L of a mixture containing 100 μ L of 80 mM PBS (pH 5.4), 85 μ L of 0.22 M PBS (pH 5.4) and 15 μ L of 0.017% H₂O₂ were added to the wells. The reaction was started by the addition of 20 μ L of 18.4 mM tetramethylbenzidine HCl (TMB) in dimethylformamide. The mixture was incubated for 3 min at 37 °C, and the reaction was subsequently stopped by the addition of 30 μ L of 1.46 M sodium acetate (pH 3.0). Enzyme activity was determined colorimetrically using a plate reader (Bio-Tek Multi-Mode Microplate reader Synergy HT) to measure absorbance at 620 nm, and the results are expressed as mili optical density (mDO) per biopsy.

2.6 TISSUE N-ACETYL- β -D-GLUCOSAMINIDASE (NAG) ASSAY

The activity of NAG was used as a marker for the presence of mononuclear leukocytes in inflamed tissue. According to the method used by Sanchez and Moreno (1999), ear samples (6 mm circles) were treated using the same method described for the MPO assay. The supernatant samples (triplicates of 25 μ L) were added into 96-well plates. For the assay, 25 μ L of p-nitrophenyl-acetamide- μ -d-glucopyranoside (2.24 mM) and 100 μ L of 50 mM buffer citrate (pH 4.5) per well were used. The mixture was incubated for 60 min at 37 °C, and the reaction was stopped by the addition of 100 μ L of 200 nM glycine buffer (pH 10.4). The enzyme activity was determined colorimetrically using a plate reader (Bio-Tek Multi-Mode Microplate reader Synergy HT) to measure absorbance at 405 nm, and enzyme activity was expressed as mDO per biopsy.

2.7 DETERMINATION OF REDUCED GLUTATHIONE (GSH) LEVELS AND LIPID HYDROPEROXIDE (LOOH) CONTENT

Ear tissue samples were homogenized 1:10 (w/v) with potassium phosphate buffer, pH 6.5. The homogenate was used to measure reduced glutathione (GSH) and hydroperoxide (LOOH) levels.

Reduced glutathione levels were determined according to the method of Sedlak and Lindsay (1968). Aliquots of tissue homogenate were mixed with 12.5% trichloroacetic acid, vortexed, and centrifuged for 15 min at 6000 \times g. The supernatant was reserved, and TRIS buffer (0.4 M, pH 8.9) and 5,5'-dithiobis 2-nitrobenzoic acid (DTNB; 0.01 M) were added. Absorbance was read at 415 nm using a spectrophotometer. The procedures were performed at 4°C, and the individual values were interpolated into a standard curve of GSH and are expressed as μ g·g of tissue⁻¹.

The levels of LOOH were determined using the ferrous oxidation-xyleneol orange (FOX2) method as described by Jiang, Hunt and Wolff (1992). Briefly, 100 μ L of methanol P.A. was added to 100 μ L of supernatant, vortexed, and centrifuged at

9700 x *g* for 5 min at 4°C. The supernatant was mixed with FOX2 reagent (4 mM butylated hydroxytoluene [BHT], 250 mM FeSO₄, 25 mM H₂SO₄, and 100 mM xylene orange) and incubated in the dark for 30 min at room temperature. Absorbance was read at 560 nm in a microplate reader, and the concentration of LOOH is expressed as mmol hydroperoxide·mg of protein⁻¹.

The protein content in ear tissue was measured to express the results of the LOOH levels. Protein concentrations of the supernatants were determined by the Bradford method (Bio-Rad, Hercules, CA, USA) using bovine serum albumin (125-1000 µg) as the standard and performed according to the manufacturer's instructions.

2.8 *IN VITRO* EVALUATION OF THP-1 CELLS ACTIVATION

THP-1 cells (Istituto Zooprofilattico di Brescia, Italy), cells were used at a density of 10⁶ cells/mL in RPMI 1640 containing 2mM L-glutamine, 0.1 mg/mL streptomycin, 100 IU/mL penicillin, 50 µM 2-mercaptoethanol, supplemented with 10% heated-inactivated fetal calf serum (media) and cultured at 37°C in 5% CO₂. For cytotoxicity or CD86 expression, 0.5 x 10⁶ cells were seeded in 24-well plates. For IL-8 mRNA expression 3.0 x 10⁶ cells were seeded in 14 mL round bottom tubes.

2.8.1 CELL VIABILITY

Cytotoxicity was assessed by flow cytometric evaluation of propidium iodide (PI)-stained cells. Cells were seeded in 24-well plates (0.5 mL/well) and incubated for 24 hours with oxazolone alone or in association with DBP, LPS (10 ng/mL) as positive control, or DMSO as vehicle control. After incubation, cells were centrifuged at 1500 rpm for 5 minutes and suspended in 500 µL PBS containing 1 µg/mL PI. The percentage of positive cells was analyzed using a FACSCalibur flow cytometer, and data were quantified using CellQuest software (BD, Becton Dickinson). Based on cell

viability, cells were treated using the concentration of chemicals resulting in cell viability > 75%.

2.8.2 FLOW CYTOMETRIC ANALYSIS OF CD86 EXPRESSION

CD86 expression was evaluated by flow cytometric analysis. THP-1 cells were treated with oxazolone, DBP, oxazolone associated with DBP, LPS or DMSO as vehicle control. After 24 hours, cells were centrifuged at 1500 rpm for 5 minutes. Cells were then stained for 30 minutes with a specific FITC-conjugated antibody against CD86 (BD, Becton Dickinson) or with isotype control antibody at 4°C following supplier's instructions. The intensity of fluorescence and the percentage of positive cells were analyzed using FACSCalibur flow cytometer, and data were quantified using CellQuest software (BD, Becton Dickinson). 10,000 viable cells were analyzed for mean fluorescence intensity (MFI) and percentage of positively labeled cells (PC). Changes in CD86 expression are reported according to Azam *et al.* (2006) as stimulation index (SI) calculated by the following equation:

$$SI = \frac{PC_t \times MFI_t}{PC_c \times MFI_c}$$

PC_c and MFI_c stand for the percentage of positive cells and mean fluorescence intensity in control cells, whereas PC_t and MFI_t stand for the percentage and mean fluorescence intensity in chemical-treated cells.

2.8.3 REAL TIME RT-PCR

Total RNA was isolated from THP-1 cells after 3 hours of treatment with selected chemicals and associations using commercial available kit (TriReagent from Sigma) as described by the manufacturer. For the synthesis of cDNA, 2.0 µg of total RNA was retro-transcribed using a high-capacity cDNA archive kit from Applied Biosystems (Foster City, CA, USA) following the supplier's instructions. IL-8 gene expression was evaluated by Real Time reverse transcription polymerase chain

reaction (Real Time PCR). For PCR-analysis, Taq-ManTM-PCR technology was used. For each PCR reaction, 10 ng of total RNA were used. The 18S ribosomal RNA transcription was used as endogenous reference and the quantification of the transcripts was performed by the $\Delta\Delta C_T$ method.

2.9 DATA ANALYSIS

The data presented are expressed as mean \pm SEM (*in vivo* experiments) or as mean \pm SD (*in vitro* experiments). Statistical significance was determined by ANOVA followed by Tukey multiple comparison tests using the GraphPad Prism software (GraphPad Software, La Jolla, CA). When necessary, Welch's t test was performed to confirm significant differences. Differences were considered significant for $p \leq 0.05$.

3 RESULTS

3.1 EFFECTS OF SENSITIZATION AND ELICITATION WITH DBP

As expected, animals sensitized and challenged with DBP did not develop contact hypersensitivity. In this scenario, DBP did not induce ear edema nor increased the infiltrated cells count, or dermal and epidermal thickness (data not shown).

3.2 EFFECTS OF DBP ON OXAZOLONE-INDUCED EAR EDEMA IN BALB/c MICE

The effect of DBP on ear edema induced by topical treatment of oxazolone is shown in Figure 2. The figure represents the evolution of ear edema 24, 48 and 72 hours after challenge with oxazolone. There was an increase in ear edema in animals treated with oxazolone (positive control group) when compared with the negative control group (challenged with vehicle). Topical treatment with DBP 0.4 and 4 mg/ear at 12-hour intervals significantly increased the edema 24 (92 and 78 % increase, respectively), 48 (72 and 83% increase, respectively) and 72 hours (65 and 104% increase, respectively) after challenge with oxazolone. DBP 0.04 mg/ear did not aggravate ear edema induced by oxazolone when compared with the positive control group.

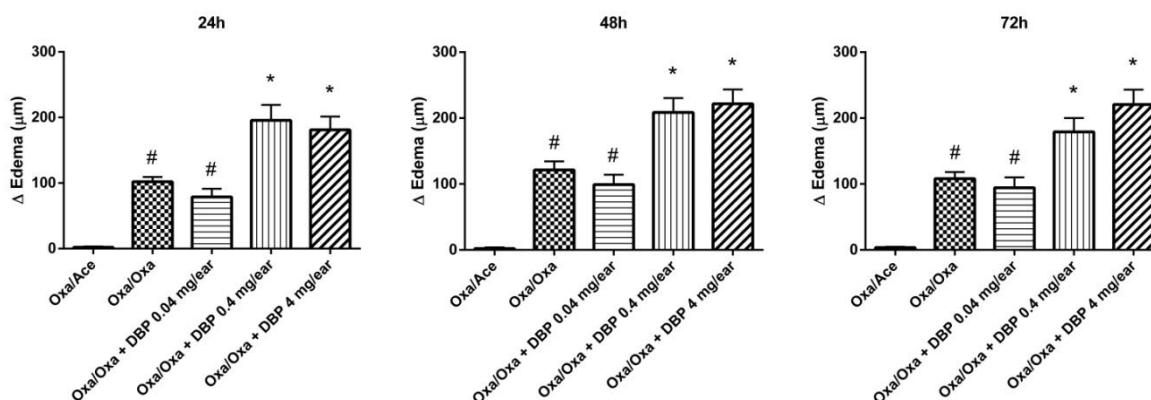


FIGURE 2. EVOLUTION OF EAR EDEMA AFTER CHALLENGE WITH OXAZOLONE AND TREATMENT WITH DIBUTYL PHTHALATE. Following abdominal sensitization with oxazolone, the right ear of the animals was challenged with vehicle (acetone) or oxazolone and then treated with vehicle or DBP. Ear thickness was measured 24, 48 and 72 hours after challenge. Sensitization/Elicitation: Oxa/Ace - oxazolone/acetone (n=6); Oxa/Oxa - oxazolone/oxazolone (n=6); Oxa/Oxa + DBP 0.04 mg/ear - oxazolone/oxazolone + DBP 0.04 mg/ear (n=5); Oxa/Oxa + DBP 0.4 mg/ear - oxazolone/oxazolone + DBP 0.4 mg/ear (n=6); Oxa/Oxa + DBP 4 mg/ear - oxazolone/oxazolone + DBP 4 mg/ear (n=6). (#) significantly different from the negative control group. (*) significantly different from both negative and positive control groups ($p < 0,05$, ANOVA followed by Tukey).

3.3 EFFECTS OF TREATMENT WITH DBP AND OXAZOLONE IN CELL INFILTRATE AND DERMAL AND EPIDERMAL THICKNESS

The topic administration of oxazolone induced the recruitment of inflammatory cells to the ear tissue as clearly demonstrated in Figure 3B. In addition, the association of oxazolone and DBP 0.4 and 4 mg/ear appear to have further increased the infiltration of inflammatory cells (FIGURE 3D and 3E, respectively). These qualitative data were confirmed by the cell count in the dermis, which revealed significant increase in cell number in the groups treated with DBP 0.4 and 4 mg/ear associated with oxazolone when compared with the positive control group (43 and 77% increase, respectively). The group treated with DBP 0.04 mg/ear did not induce significant increase in the number of cells in the dermis when compared to the positive control group (FIGURE 4).

Dermal thickness was increased in animals sensitized and challenged with oxazolone when compared with the negative control group. In addition, animals treated with DBP 0.4 and 4 mg, but not 0.04 mg, presented increased dermal thickness in comparison with the positive control group (38 and 69% increase, respectively) (FIGURE 4).

The epidermis of ears treated with oxazolone associated to DBP was measured to evaluate the effect of DBP on epidermal hyperplasia induced by oxazolone. Results are shown in Figures 3 and 4. An increase in epidermal cells in the positive control group and the groups treated with oxazolone associated to DBP can be observed in Figure 3. As it is shown in Figure 4, there was significant difference between the negative and positive control groups, and between the groups treated with DBP in the two higher doses and the positive control group (74 and 84%

increase induced by DBP 0.4 and 4 mg, respectively). DBP 0.04 mg/ear did not increase the epidermal hyperplasia induced by oxazolone.

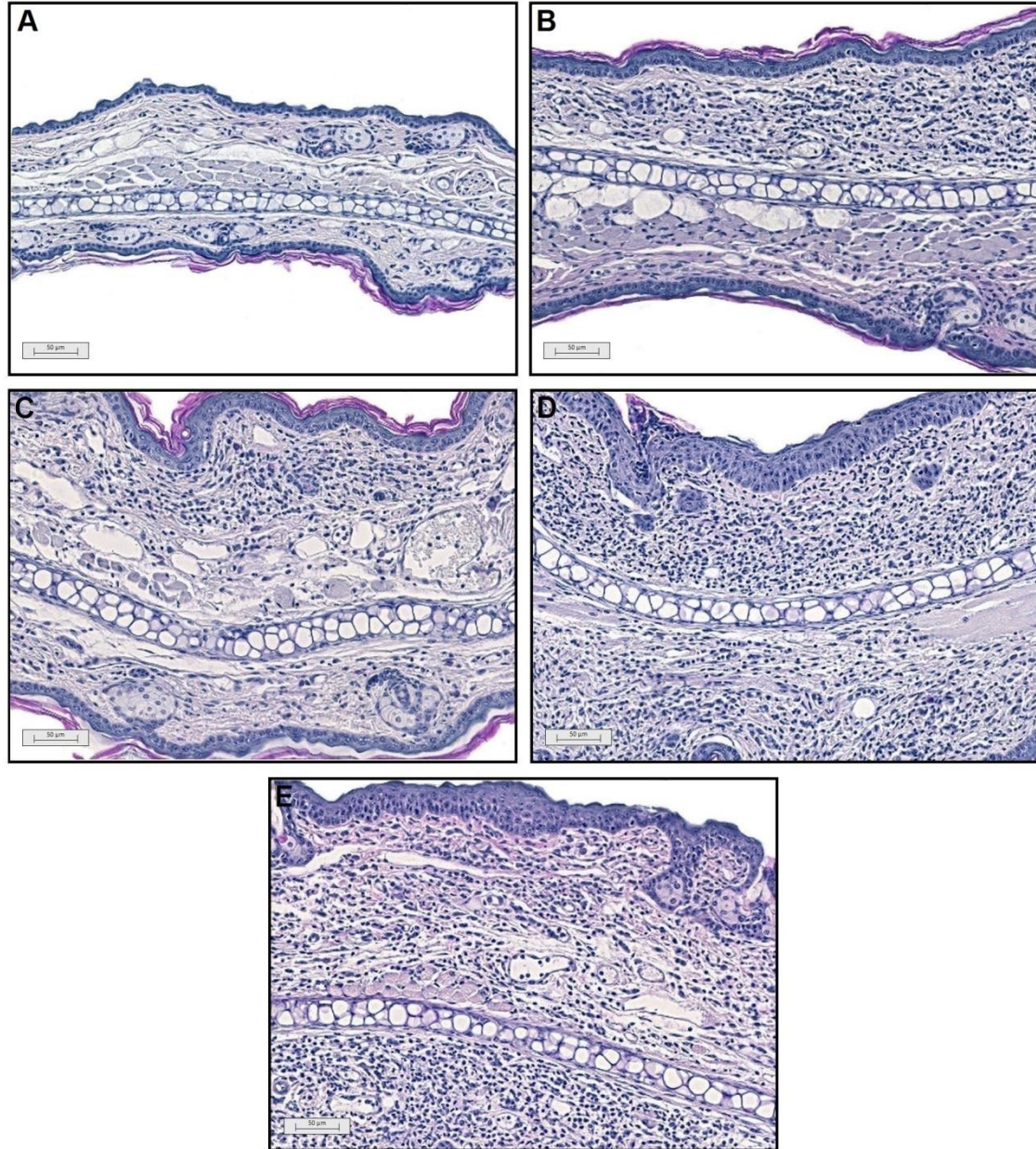


FIGURE 3. PHOTOMICROGRAPHS OF THE RIGHT EAR OF REPRESENTATIVE ANIMALS FROM ALL GROUPS (100X). Following abdominal sensitization with oxazolone, the right ear of the animals was challenged with vehicle or oxazolone and then treated with vehicle or DBP. Sensitization/Elicitation. (A) oxazolone/acetone; (B) oxazolone/oxazolone; (C) oxazolone/oxazolone + DBP 0.04 mg/ear; (D) oxazolone/oxazolone + DBP 0.4 mg/ear; (E) oxazolone/ oxazolone + DBP 4 mg/ear.

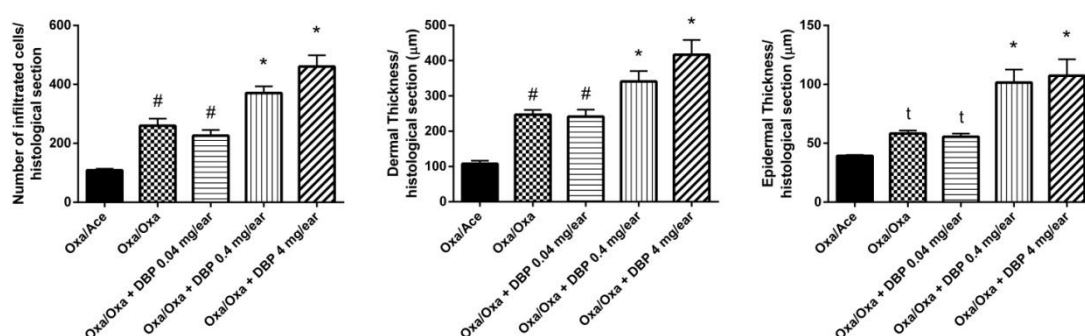


FIGURE 4. HISTOLOGICAL PARAMETERS. Following abdominal sensitization with oxazolone, the right ear of the animals was challenged with vehicle (acetone) or oxazolone and then treated with vehicle or DBP. Sensitization/Elicitation: Oxa/Ace - oxazolone/acetone (n=6); Oxa/Oxa - oxazolone/oxazolone (n=6); Oxa/Oxa + DBP 0.04 mg/ear - oxazolone/oxazolone + DBP 0.04 mg/ear (n=5); Oxa/Oxa + DBP 0.4 mg/ear - oxazolone/oxazolone + DBP 0.4 mg/ear (n=6); Oxa/Oxa + DBP 4 mg/ear - oxazolone/oxazolone + DBP 4 mg/ear (n=6). (#) significantly different from the negative control group; (*) significantly different from both negative and positive control groups ($p < 0.05$, ANOVA followed by Tukey); t: significantly different from the negative control group ($p < 0.05$, Welch's t test).

3.4 EFFECTS OF OXAZOLONE AND DBP IN TISSUE MYELOPEROXIDASE (MPO) AND N-ACETYL- β -D-GLUCOSAMINIDASE (NAG) ACTIVITY

The effect of topical treatment with DBP associated to oxazolone on MPO and NAG activity is reported in Figure 5. Oxazolone increased MPO activity in comparison with the negative control group. This increase was significantly higher in the groups treated with DBP 0.4 and 4 mg/ear when compared with the positive control group.

DBP 4 mg/ear, associated to oxazolone, was able to induce significant increase in NAG activity induced by oxazolone. DBP 0.4 mg/ear induced a slight increase when compared with the positive control group, but this increase was not significant. On the contrary, DBP 0.04 mg/ear reduced NAG activity induced by oxazolone.

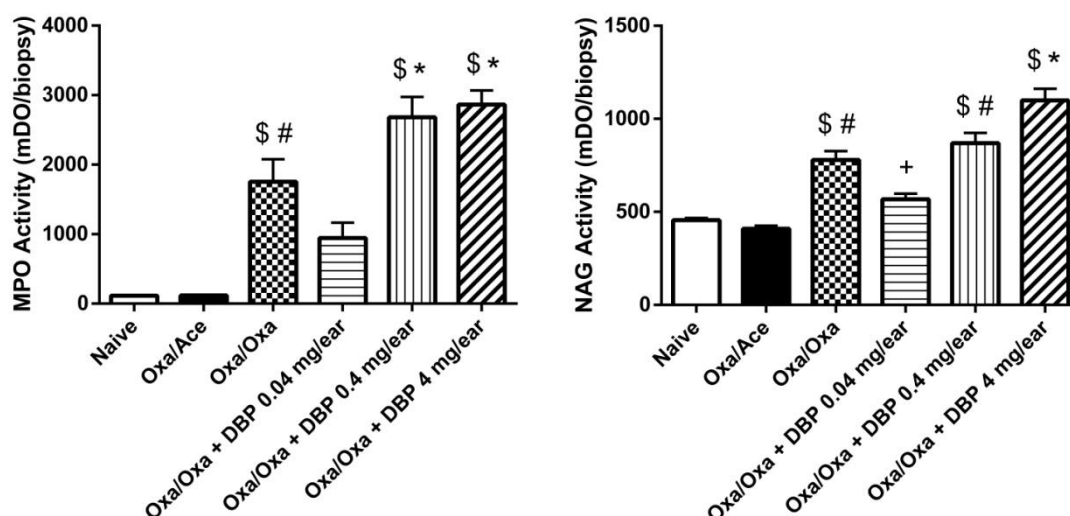


FIGURE 5. EFFECTS OF EXPOSURE TO DBP ASSOCIATED TO OXAZOLONE ON MPO AND NAG ACTIVITY. Following abdominal sensitization with oxazolone, the right ear of the animals was challenged with vehicle (acetone) or oxazolone and then treated with vehicle or DBP. Naïve: animals were not exposed to any substance. Sensitization/Elicitation. Oxa/Ace - oxazolone/acetone (n=6); Oxa/Oxa - oxazolone/oxazolone (n=6); Oxa/Oxa + DBP 0.04 mg/ear - oxazolone/oxazolone + DBP 0.04 mg/ear (n=6); Oxa/Oxa + DBP 0.4 mg/ear - oxazolone/oxazolone + DBP 0.4 mg/ear (n=7); Oxa/Oxa + DBP 4 mg/ear - oxazolone/oxazolone + DBP 4.0 mg/ear (n=7). (\$) significantly different from naïve group. (#) significantly different from the negative control group. (+) significantly different from positive control group. (*) significantly different from both negative and positive control groups. $p < 0.05$, ANOVA followed by Tukey.

3.5 EFFECTS OF OXAZOLONE AND DBP ON LEVELS OF REDUCED GLUTATHIONE (GSH) AND LIPID PEROXIDATION (LPO)

Both negative and positive control groups induced a reduction in GSH levels when compared with the naïve group. This reduction was more pronounced in the positive control group. The highest dose of DBP (4 mg/ear) did not differ from the positive control group. DBP 0.4 mg/ear induced a slight reversion in the GSH reduction induced by oxazolone in the positive control group, but it was not different from the negative control group. However, DBP 0.04 mg/ear completely reversed the reduction in GSH observed in the positive and negative control groups, therefore not being different from the naïve group (FIGURE 6).

Increase in LOOH levels was observed in the positive control group in comparison with naïve and negative control groups. The highest dose of DBP was not different from the positive control group. On the contrary, DBP 0.4 and 0.04 mg/ear reduced significantly the levels of LOOH observed in the positive control

group. The reduction induced by DBP 0.04 mg/ear was more evident since this group was not different from naïve and negative control groups (FIGURE 6).

Since phthalates have been related to oxidative stress, we also evaluated these parameters in the animals sensitized and challenged with DBP 0.4 mg. DBP induced the reduction of GSH in comparison to the naïve group, and did not differ from the negative and positive control groups nor from the groups treated with DBP 0.4 and 4 mg/ear following oxazolone elicitation (FIGURE 6). However, DBP did not induce increase in LOOH levels, and this group was not different from the naïve and negative control groups (FIGURE 6).

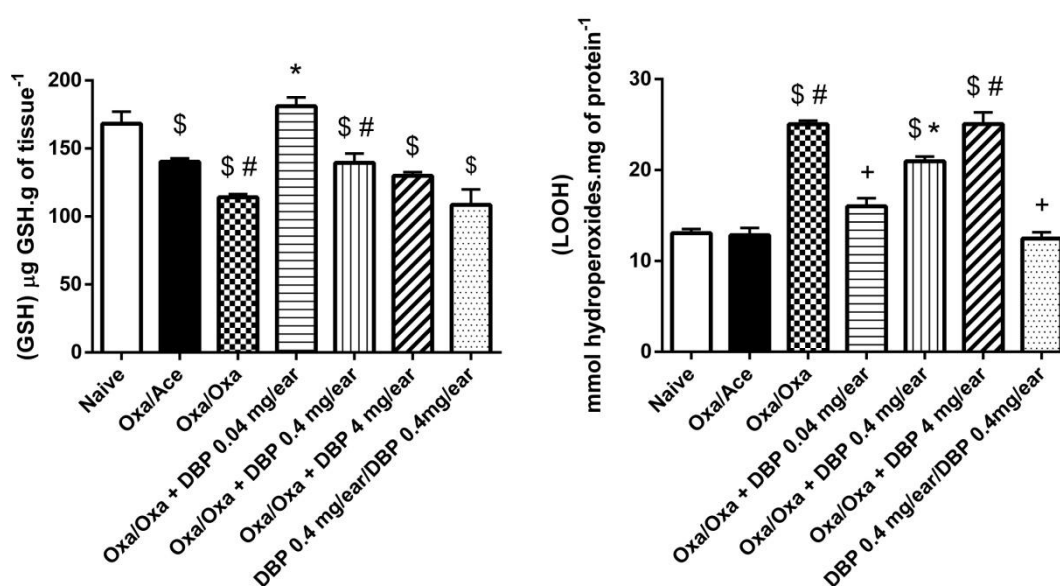


FIGURE 6. REDUCED GLUTATHIONE (GSH) AND LIPID HYDROPEROXIDE (LOOH) LEVELS. Following abdominal sensitization with oxazolone or DBP, the right ear of the animals was challenged with vehicle (acetone), oxazolone or dibutyl phthalate and then treated with vehicle or DBP. Naïve: animals were not exposed to any substance. Sensitization/Elicitation. Oxa/Ace - oxazolone/acetone (n=6); Oxa/Oxa - oxazolone/oxazolone (n=6); Oxa/Oxa + DBP 0.04 mg/ear - oxazolone/oxazolone + DBP 0.04 mg/ear (n=6); Oxa/Oxa + DBP 0.4 mg/ear - oxazolone/oxazolone + DBP 0.4 mg/ear (n=6); Oxa/Oxa + DBP 4 mg/ear - oxazolone/ oxazolone + DBP 4.0 mg/ear (n=6). DBP 0.4 mg/ear/ DBP 0.4 mg/ear (n=4). (\$) significantly different from naïve group. (#) significantly different from the negative control group. (+) significantly different from positive control group. (*) significantly different from both negative and positive control groups. $p < 0.05$, ANOVA followed by Tukey.

3.6 *IN VITRO* EVALUATION OF THP-1 CELLS ACTIVATION

For the evaluation of allergen-induced THP-1 cells activation, we assessed the expression of CD86 and IL-8 mRNA after THP-1 cells were exposed to oxazolone and DBP.

THP-1 cells were treated with increasing concentrations of the selected chemicals and associations for 24 hours. Cell viability was then assessed by flow cytometry using PI staining. According to the results, based on cell viability, cells were then treated with concentrations of chemicals resulting in more than 75% of viability, which corresponded to 40 µg/mL of oxazolone and 250, 500 and 1000 µM of DBP.

THP-1 cells were first treated for 24 hours with the selected concentrations of DBP. None of the doses of DBP were able to induce the expression of CD86 (data not shown). Exposure to oxazolone showed an increase in the expression of CD86, which was significantly higher in the groups exposed to the associations between oxazolone with the two highest doses of DBP (FIGURE 7).

Oxazolone induced the expression of IL-8 mRNA, and, surprisingly, DBP was also capable of inducing this expression. No difference was found in the group treated with both oxazolone and DBP when compared to the group treated with oxazolone (FIGURE 7).

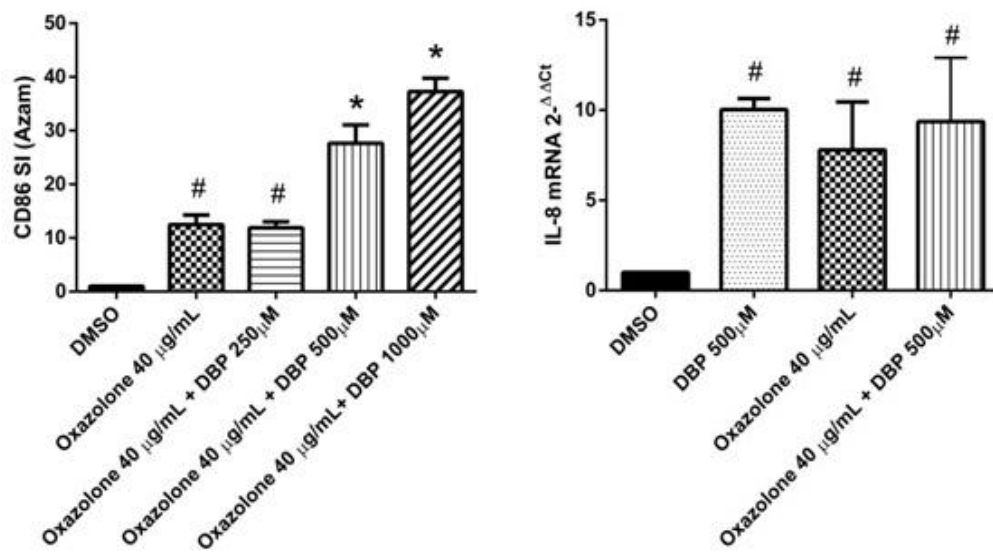


FIGURE 7. EFFECTS OF OXAZOLONE AND ASSOCIATION WITH DBP ON THE EXPRESSION OF CELL SURFACE MARKER CD86 AND IL-8 mRNA. For CD86 expression, THP-1 cells were treated for 24h with oxazolone or its association with increasing doses of Dibutyl phthalate (DBP). Vehicle treated cells were included as a vehicle control (DMSO). Results are expressed as stimulation index (SI). Each value represents the mean \pm SD, $n=3$. For IL-8 mRNA, THP-1 cells were treated for 3 h with oxazolone, DBP or the association between oxazolone and DBP. Vehicle treated cells were included as a vehicle control (DMSO). IL-8 mRNA expression was evaluated by Real Time-PCR as described in the Material and Methods section. Results are expressed as $2^{-\Delta\Delta C_T}$. Each value represents the mean \pm SD, $n=3$ independent experiments. (#) significantly different from the negative control group; (*) significantly different from both negative control group and oxazolone group. $p<0.05$, ANOVA followed by Tukey multiple comparison test.

4 DISCUSSION

Since humans are commonly exposed to phthalates, it is important to elucidate their potential immunotoxic effects. Toxicological studies have demonstrated that phthalate esters, like DBP, do not possess significant potential to cause sensitization on skin. This is because these molecules are not capable of bonding and reacting to proteins, which is mandatory for the allergic stimulation by a substance (KIMBER; DEARMAN, 2010). However, phthalate esters have been reported to facilitate the development of allergic diseases such as asthma and atopic dermatitis, and to act as adjuvants in animal models of contact hypersensitivity (IMAI *et al.*, 2006; MATSUDA *et al.*, 2010). The present *in vivo* study investigated the effects of DBP on an animal model of contact hypersensitivity induced by oxazolone in BALB/c mice. Results indicate that DBP aggravates the inflammatory response involved in this model.

DBP, in the two highest doses (0.4 and 4 mg/ear) was able to increase the ear edema and dermal thickness induced by oxazolone in mice, besides enhancing the leukocyte recruitment to the dermis, as indicated by cell count and MPO and NAG activity. Added to that, the thickness of the epidermis was significantly different between the group treated with oxazolone and those associated with the two highest doses of DBP (0.4 and 4 mg/ear). These results indicate that DBP can aggravate several pathophysiological signs that characterize the oxazolone-induced model of contact hypersensitivity in BALB/c mice. In addition to a persistent edema, the infiltration of inflammatory cells and epidermal hyperplasia are typical features of this animal model (FUJII *et al.*, 2002).

The increase in ear edema (IMAI *et al.*, 2006; SHIBA *et al.*, 2009; MATSUDA *et al.*, 2010; LI *et al.*, 2014) and cell infiltration (TAKANO *et al.*, 2006; LI *et al.*, 2014) demonstrated in this study had already been reported by other authors. Imai *et al.* (2006) demonstrated that, not only DBP, but also other phthalates, were capable of exacerbating the ear edema induced by fluorescein isothiocyanate. Takano *et al.* (2006) showed increase in ear edema and also cell infiltration to the inflammatory site in an animal model of atopic dermatitis where animals were exposed to DEHP (di-2-ethylhexyl phthalate). Moreover, Li *et al.* (2014) showed that prolonged dermal exposure to DBP (4 and 40 mg/kg/day for forty days) was capable of increasing ear

edema and leukocyte infiltration to the dermis. The data presented here further support the potential for adjuvancy of DBP, but we report an adjuvant effect after short term dermal exposure to lower doses of DBP. Besides, our results indicate that DBP can act as adjuvant even after the sensitization has already been established, modulating the elicitation phase of oxazolone-induced CHS. Previous studies have usually observed this effect in animal models of CHS associating phthalates in both sensitization and elicitation phases, or with high doses of phthalates, long term exposure to lower doses or in different routes of exposure, in some cases with subcutaneous or even intraperitoneal injections, which are not routes by which humans would normally encounter phthalates in domestic or occupational environment.

Oxazolone is highly used in contact hypersensitivity models for promoting the phases of sensitization and elicitation. However, the great majority of studies evaluate the effects caused by the application of these substances in lymph nodes and epidermis, and little is discussed about the inflammatory properties in the dermis (ZHANG; TINKLE, 2000). The evaluation of the activity of NAG and MPO was performed to indicate the presence of mononuclear and polymorphonuclear leukocytes, respectively, in the inflamed tissue (SANCHEZ; MORENO, 1999; BRADLEY *et al.*, 1982), and characterize the probable profile of recruited cells to the dermis. The results show that both polymorphonuclear (mainly neutrophils) and mononuclear leukocytes (mainly macrophages) are present in the inflammation induced by oxazolone. These results corroborate the findings of Wiedow *et al.* (1992), who quantified macrophages and neutrophils' proteases in the dermis, which were increased in allergic contact dermatitis. DBP 0.4 increased the activity of MPO and DBP 4 mg/ear increased the activity of both enzymes, indicating a greater recruitment of cells to the inflammatory site. We can also suggest that as result of the adjuvant effect of DBP a higher proportion of neutrophils are recruited to the inflammatory site in relation to mononuclear leukocytes, since the treatment with DBP led to a higher increase in the activity of MPO than NAG. Neutrophils are involved in both sensitization and elicitation phases of CHS, and are one of the first cells to be recruited after the elicitation in sensitized animals (DILULIO *et al.*, 1999). Zhang and Tinkle (2000) found that neutrophils are found in higher amount in the dermis of mice treated with oxazolone and remain dominant until 72 hours after treatment. Besides, Tanaka *et al.* (2012) showed a much higher increase in neutrophils rather than

macrophages induced by adjuvant effect of DEHP on allergen ovalbumin-induced peritonitis in ICR mice. In this study DBP increased THP-1 oxazolone-induced activation, as showed by increased CD86 expression. Even though we did not identify the same adjuvant effect in IL-8 mRNA expression, we have previously reported that DBP enhanced IL-8 mRNA expression as well as IL-8 release in THP-1 cells exposed to citral and imidazolidinyl urea (LOURENÇO *et al.*, 2015) supporting the hypothesis that DBP can potentiate THP-1 allergen-induced activation. Therefore, DBP could be enhancing the activation of dendritic cells *in vivo*, which could lead to higher production of IL-8 (a potent neutrophil chemoattractant) (BARKER *et al.*, 1991) and this could be reflected in higher neutrophil recruitment as part of the mechanism of DBP's adjuvant effect. Interestingly, DBP also induced a significant expression of IL-8 mRNA by itself. In general, little can be found in literature relating DBP exposure and increase in pro-inflammatory cytokines mRNA expression, therefore, this induction needs further investigation.

Although many studies on adjuvant effect of phthalates have been performed, little is known about the molecular mechanisms involved in such effect. Evidence suggests that oxidative stress could play a role in allergic and inflammatory skin diseases (OKAYAMA, 2005; BYAMBA *et al.*, 2010; MIZUASHI *et al.*, 2005). Several authors have reported that some phthalates induce oxidative stress demonstrating increased generation of reactive oxygen species (ROS) and serum markers of inflammation and oxidative stress after exposure to phthalates (SEO *et al.*, 2004; HAUSER *et al.*, 2007; FERGUSON; LOCH-CARUSO; MEEKER, 2011; TETZ *et al.*, 2013). We have previously observed a slight increase in ROS production induced by 500 μ M of DBP in the human promyelocytic cell line THP-1 (a surrogate for dendritic cell activation model). Additionally, we reported that DBP significantly increased the production of ROS induced by the allergen citral, suggesting that the adjuvant effect of DBP could be through dendritic cell activation and that this could be triggered by increase in oxidative stress (LOURENÇO *et al.*, 2015). In this study, we showed a decrease in GSH levels induced by oxazolone in the positive control group and also in the negative control group, which was exposed to oxazolone in only one phase of the sensitization model. This reduction was accompanied by increase in LOOH levels in the positive control group. The oxidative stress induced by oxazolone in contact hypersensitivity has already been reported (BRUNYÁNSZKI *et al.*, 2010). We also show that the sensitization and challenge with DBP 0.4 mg

reduced the levels of GSH, but no increase in LOOH was observed in this group. What is interesting is that the same dose (0.4 mg) partially reversed the reduction in GSH and increase in LOOH induced by oxazolone, while DBP 0.04 mg completely reversed the same parameters, and the group treated with DBP 4 mg did not differ from the positive control group. Overall, our results seem to indicate that although DBP may alter some oxidative stress parameters, these mechanisms do not participate in the adjuvant effect of DBP at the dose levels tested. Accordingly, Chen *et al.* (2015) reported a weak adjuvant effect of DBP in a murine asthma model and that this effect was not mediated through an oxidative stress mechanism. Although we have reported that DBP could induce the production of ROS *in vitro* and increase this production induced by an allergen, the time of evaluation of that parameter was different from what was performed in the present study. *In vitro* we evaluated the production of ROS immediately after the treatment of the cells with DBP associated to allergen; on the other hand, here, GSH and LOOH were measured after three days of exposure to DBP associated with oxazolone, so we should also consider the role of the inflammatory response induced by both DBP and oxazolone in such effects. In addition, phthalates tend to present opposite effects in studies about their adjuvancy when using low or high doses, different times and routes of exposure (KIMBER; DEARMAN, 2010); therefore different doses of phthalates could possibly influence the cells' redox balance in different ways.

Even though the doses 0.4 and 4 mg of DBP presented adjuvant effect in this study, the lowest dose (0.04 mg) did not differ from the positive control group in most of the parameters analyzed, besides reducing significantly NAG and MPO activity, and reversing the oxidative stress parameters. Although the majority of data found in literature reports an adjuvant effect induced by phthalates, some authors have reported a negative adjuvant effect (DEARMAN *et al.*, 2008; CHEN *et al.*, 2015). Furthermore, it has been reported that some phthalates in certain doses display what were described as "immunosuppressive" effects (inhibition of IgG1 and/or IgE antibody production) (LARSEN *et al.*, 2001). It's also been suggested that the adjuvant activity of phthalates seems to be highly dependent of the length of the alkyl side chain, giving rise to maximum adjuvant effect by phthalates with 8 or 9 carbon atoms in their alkyl chains, like DEHP, but not DBP (LARSEN; NIELSEN, 2008).

In conclusion, we suggest that DBP presents an *in vivo* adjuvant effect when associated with oxazolone in much lower doses and time of exposure than those

usually seen in animal models of contact hypersensitivity, supporting previous data of this effect in allergic diseases. However, the mechanisms underlying these effects and under what specific conditions (doses, time and route of exposure) phthalates are able to effect a meaningful change in immune function have not been fully understood, thus, future studies are necessary to elucidate the effects of phthalates *in vivo*.

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5.3.1 Material Complementar

5.3.1.1 Highlights

We report adjuvant effect of dibutyl phthalate in oxazolone-induced contact hypersensitivity.

We observed increased ear edema, leukocyte recruitment and epidermal hyperplasia.

Increase in neutrophils seems to be part of the adjuvant effect mechanism.

Oxidative stress does not seem to play a role in the mechanism of this adjuvant effect.

Increased dendritic cell activation seems to be part of the mechanisms underlying the adjuvant effect.

5.3.1.2 Graphic Abstract

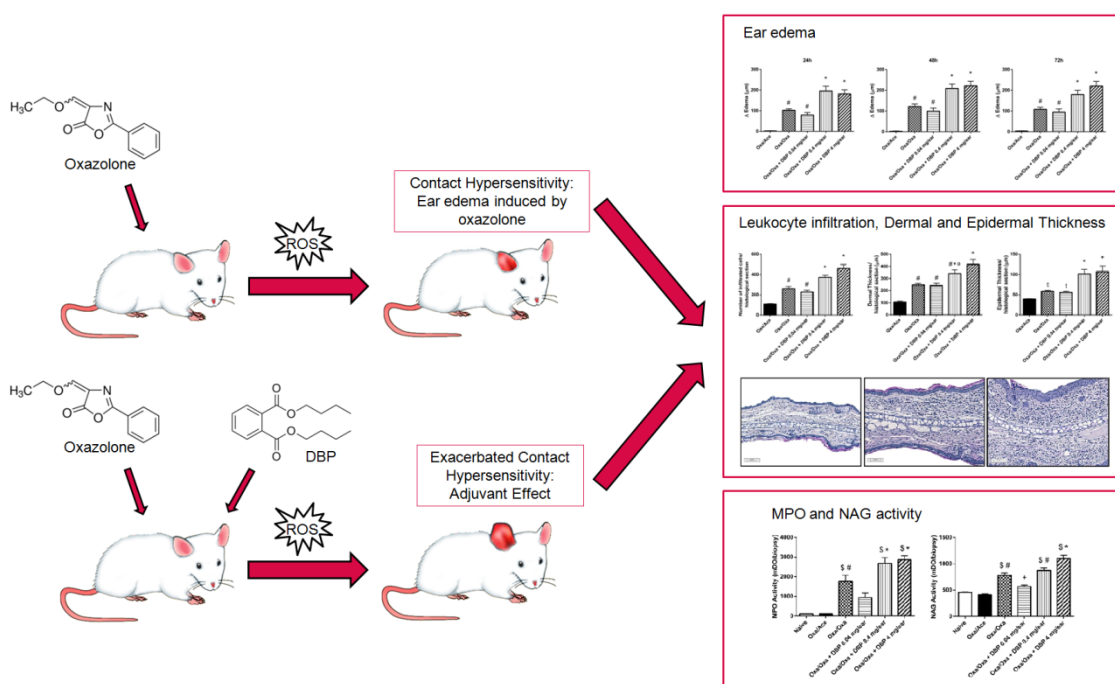


FIGURA 09. GRAPHIC ABSTRACT.

5.3.1.3 Figuras complementares

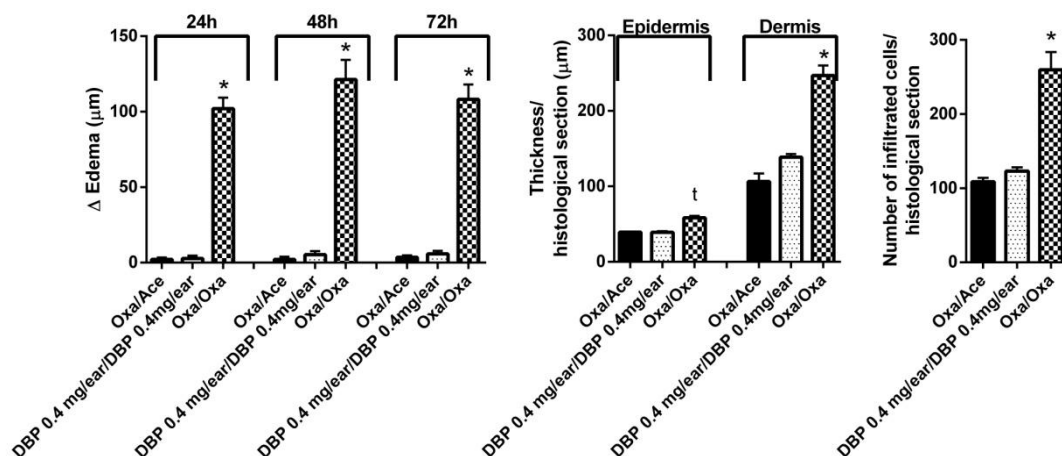


FIGURA 10. EFEITOS DO DIBUTIL FTALATO SOBRE EDEMA DE ORELHA E PARÂMETROS HISTOLÓGICOS. Após sensibilização abdominal, a orelha direita dos animais foi desafiada com veículo (acetona), oxazolona ou dibutil ftalato (DBP). A espessura da orelha foi mensurada 24, 48 e 72 horas após o desafio. Para cada animal, parâmetros histológicos foram avaliados em três campos de três cortes. Sensibilização/Desafio: Oxa/Ace (oxazolona/acetona, controle negativo), DBP 0.4 mg/ear/DBP 0.4 mg/ear (DBP 0.4 mg/DBP 0.4 mg), Oxa/Oxa (oxazolona/oxazolona, grupo controle positivo). (*) diferente do grupo controle negativo e do grupo DBP/DBP. $p < 0.05$, ANOVA seguido de Tukey. (t) diferente do grupo controle negativo. $p < 0.05$, teste t com correção de Welch.

5.4 ARTIGO 3

The involvement of transient receptor potential A1 (TRPA1) and V1 (TRPV1) activation in the adjuvant effect of dibutyl phthalate in oxazolone-induced contact hypersensitivity in BALB/c mice.

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ABSTRACT

Contact hypersensitivity (CHS) is an allergic reaction resulting from repeated contact with chemicals called haptens, consisted in two phases: sensitization and elicitation. The plasticizer dibutyl phthalate (DBP) has been shown to enhance the inflammatory response in allergen-induced CHS when associated both in sensitization and elicitation phases, however the molecular mechanisms involved in this adjuvant effect are not fully understood. The present study aimed to investigate the possible participation of transient receptor potential cation channel (TRP) A1 (TRPA1) and V1 (TRPV1) activation by DBP in an animal model of CHS induced by oxazolone in BALB/c mice. For the experiments, adult male BALB/c mice were sensitized (in shaved abdomen) and challenged (elicitation; in the right ear) with oxazolone or vehicle (acetone) topically, and subsequently treated 6 times (with 12 hour intervals) with vehicle, TRPA1 or TRPV1 antagonists (HC030031 or SB366791, respectively) followed by treatment with DBP or vehicle (topically). Ear thickness was measured before elicitation (baseline) and 24, 48 and 72 hours after elicitation to calculate ear edema. After the last measure, animals were euthanized and 6 mm circles of ear tissue were collected to evaluate the activity of the enzymes N-acetyl- β -d-glucosaminidase (NAG) and Myeloperoxidase (MPO) (markers of leukocyte infiltration) and histological analysis (infiltrated cell count, dermal and epidermal thickness). Oxazolone induced CHS (increased ear edema, leukocyte infiltration and epidermal hyperplasia) and DBP increased all of these parameters confirming its adjuvant effect. Animals presenting CHS treated with TRPA1 antagonists and DBP showed reduction in the analyzed parameters when compared to those treated only with DBP, but this group was still different from its positive control group. Treatment with TRPV1 antagonist completely reversed the parameters to positive control group levels in animals with CHS treated with TRPV1 antagonist and DBP. In conclusion we suggest that the activation of TRPV1 and TRPA1 by DBP in the elicitation phase of oxazolone-induced CHS could be part of the mechanism of DBP's adjuvant effect, and that TRPV1 activation seems to be more important in this adjuvant effect during the elicitation phase. Our results contribute to further understanding of the mechanisms involved in the adjuvant effect of phthalates in immune responses.

Key words: dibutyl phthalate, contact hypersensitivity, oxazolone, elicitation, TRPA1, TRPV1.

1 INTRODUCTION

Allergic contact dermatitis (ACD) or contact hypersensitivity (CHS) is a delayed type hypersensitivity reaction mediated by antigen specific T lymphocytes (KARLBERG *et al.*, 2008) in response to repeated or prolonged contact to chemical substances called haptens (HENNINO *et al.*, 2005; KARLBERG *et al.*, 2008). There are two phases involved in CHS: the sensitization phase (first contact with the hapten, antigen presenting to the lymph node and activation of specific T lymphocytes) and the elicitation phase (subsequent contacts with hapten, recognition by specific T lymphocytes and development of inflammatory response) (GRABBE; SCHWARZ, 1998; KIMBER, 2002; HENNINO *et al.*, 2005; KARLBERG *et al.*, 2008). Several chemicals can induce CHS after repeated exposure to the skin. Although many studies have focused on chemicals exhibiting sensitizing potential, environmental chemicals with adjuvant effects may also play a role in the development of hypersensitivity reactions.

Phthalate esters, industrial chemicals mainly used to impart flexibility to polyvinyl chloride plastics, are candidate chemicals with adjuvant effect. These substances are often found in a variety of manufactured products such as plastics for food packing, glues, paints, solvents, children's products, insect repellents, besides different kinds of cosmetics, like perfumes, moisturizers, hairsprays and nail polishes (IPCS, 1997; SATHYANARAYANA *et al.*, 2008). It is been reported that, even though phthalate esters are not capable of inducing hypersensitivity themselves (ELSISI; CARTER; SIPES, 1989; DOAN; BRONAUGH; YOURICK, 2010; BEYDON; PAYAN; GRANDCLAUDE, 2010; CPSC, 2010; PAN *et al.*, 2014), certain compounds, such as dibutyl phthalate (DBP), could be able to exacerbate the potency of allergens *in vitro* and *in vivo*, and, so, play a role in the development and/or aggravation of allergic diseases (CASILLAS *et al.*, 1999, CHOWDHURY AND STATHAM, 2002; IMAI *et al.*, 2006; MATSUDA *et al.*, 2010; LI *et al.*, 2014, LOURENÇO *et al.*, 2015). However, the mechanisms underlying the adjuvant effects of phthalates are not yet fully understood.

There is evidence that the immune system presents connection with the peripheral nerve system (BERESFORD *et al.*, 2004; LIU *et al.*, 2006). It has been suggested that sensory neurons that detect noxious stimuli could be involved in the

initiation of some allergies (PALM *et al.*, 2012). It's been reported that CHS can be suppressed through systemic deletion of capsaicin (CAP)-sensitive fibers (BERESFORD *et al.*, 2004). These fibers express the transient receptor potential (TRP) V1 (TRPV1), a calcium permeable cation channel involved in nociception caused by chemical and thermal stimuli, also known as CAP receptor (CATERINA *et al.*, 1997; CLAPHAM, 2003). It's been demonstrated that some TRPV1-expressing sensory neurons could also express TRPA1, another member of the TRP family of calcium permeable cation channels (STORY *et al.*, 2003), which can be activated by chemicals such as allyl isothiocyanate (AITC) and cinnamaldehyde (BANDELL *et al.*, 2004; JORDT *et al.*, 2004). The activation of TRPA1 or TRPV1 may result in release of neuropeptides from peripheral nerve endings (BAUTISTA *et al.*, 2005; ZYGMUNT *et al.*, 1999), such as calcitonin gene-related peptide (CGRP) and substance P (SP), that have been shown to participate in the inflammatory response of CHS (MARUYAMA *et al.*, 2007; MIKAMI *et al.*, 2011; LIU *et al.*, 2013). Moreover, it's been shown that desensitization of TRPA1 and TRPV1 after local pretreatment with AITC and CAP, respectively, suppressed the sensitization to fluorescein isothiocyanate (FITC) in BALB/c mice (MARUYAMA *et al.*, 2007).

The ability of DBP to stimulate sensory neurons was demonstrated by the activation of nerve cells isolated from mouse dorsal root ganglia (SHIBA *et al.*, 2009). In this study, DBP activated TRPA1 and TRPV1, which was shown by evocation of calcium influx to TRPA1-expressing CHO cells and TRPV1-expressing CHO and HEK 293 cells. In addition, Shiba *et al.* (2012) reported that the TRPA1 antagonist HC030031 inhibited DBP-induced calcium influx into TRPA1-expressing CHO cells.

Therefore, the present study aimed to investigate the possible participation of TRPA1 and TRPV1 activation by DBP during the elicitation phase of an animal model of contact hypersensitivity induced by oxazolone in BALB/c mice. This may contribute to further understanding of the cellular mechanisms underlying the adjuvant effect of phthalates in immune responses.

2 MATERIAL AND METHODS

2.1 ANIMALS

Adult male BALB/c mice obtained from Fundação Oswaldo Cruz (FIOCRUZ – Rio de Janeiro, RJ, Brazil) were used for the experiments. The animals were kept in the animal facilities of the Environmental Toxicology Laboratory (Escola Nacional de Saúde Pública – ENSP, FIOCRUZ, Rio de Janeiro, RJ, Brazil) under a 12 h light/dark cycle and controlled temperature ($22 \pm 2^\circ\text{C}$). Standard pellet food and tap water were provided *ad libitum*. The experiments were conducted in accordance with the orientations for laboratory animal care. The experimental protocol was approved by the Committee on Animal Research and Ethics of the Universidade Federal do Paraná (Curitiba, Brazil) under the number 633.

2.2 CHEMICALS AND DOSE SELECTION

Dibutyl phthalate (DBP, CAS number 84-74-2, purity 99%), Oxazolone (4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one, CAS number 15646-46-5), the vanilloid receptor-1 (TRPV1) antagonist, SB366791, and the selective transient receptor potential cation channel (TRPA1) blocker, HC030031, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Oxazolone was selected as skin sensitizer for being frequently used to promote the phases of sensitization and elicitation of contact hypersensitivity. Oxazolone is considered a strong hapten, with potent pro-inflammatory properties (ZHANG; TINKLE, 2000). Antagonists, DBP and oxazolone were diluted in acetone (vehicle).

DBP doses were based on available data on DBP concentrations on personal care products, in particular perfumes. According to Koo and Lee (2004), the average concentration of DBP found in a variety of perfumes is 0.44 mg/mL, and the average use of perfume by the general population, estimated through an applied questionnaire, is 1mL/day. Therefore, the amount of DBP applied to the skin in

individuals that use determined kinds of perfumes can be approximately 0.4 mg/day, which corresponds to 0.0067 mg/kg/day for a 60 kg individual or 0.25 mg/m²/day using the conversion factor recommended by the FDA (FDA, 2005). These estimations are in agreement with the data published by the International Journal of Toxicology (2005) in an annual review on safety of ingredients from cosmetic products, indicating that the use of perfumes may result in skin applications of up to 0.47 mg DBP/day. Therefore, we selected the same nominal human dose of 0.4 mg/day of DBP, which corresponds to 20 mg/kg/day in a 0.02 kg mouse or 60 mg/m²/day using the conversion factor recommended for mice by the FDA publication (FDA, 2005), i.e., 240 times higher than the estimated human exposure described above (0.25 mg/m²/day).

The doses of the antagonists were selected according to the literature (SILVA *et al.*, 2011). The lowest dose with positive effect for antagonism (30 nmol/ear) was chosen for both antagonists.

2.3 EVALUATION OF EAR EDEMA

Mice were sensitized with 30 µL of oxazolone (0.25% w/v in acetone) on day 0 on shaven abdomen. After five days, baseline ear thickness was measured with a digital micrometer (Great MT-04513). Animals were then challenged on the right ear with 15 µL of oxazolone (0.5% w/v in acetone) or vehicle (elicitation). After elicitation, the right ear of the animals was treated with 20 µL of vehicle or one of the TRP antagonists (SB366791 – 30 nmol/ear; HC030031 – 30 nmol/ear). After 15 minutes, the same ear was treated with vehicle or DBP (0.4 mg/ear). The treatment with vehicle or antagonists followed by treatment with vehicle or DBP was performed 5 other times in 12 hour intervals, totalizing 6 treatments by the end of 72 hours. Ear thickness was measured 24, 48 and 72 hours after elicitation (RECIO *et al.*, 2000). Ear edema was expressed as the increase of ear thickness related to the baseline measurement in micrometers (µm). After ear edema evaluation the animals were euthanized by cervical dislocation and 6 mm circles of ear tissue were collected for posterior histological evaluation and enzyme activity assays (N-acetyl-beta-D-glucosaminidase - NAG and myeloperoxidase - MPO).

The animals were randomly divided in groups and the treatments were performed as described in Figure 1.

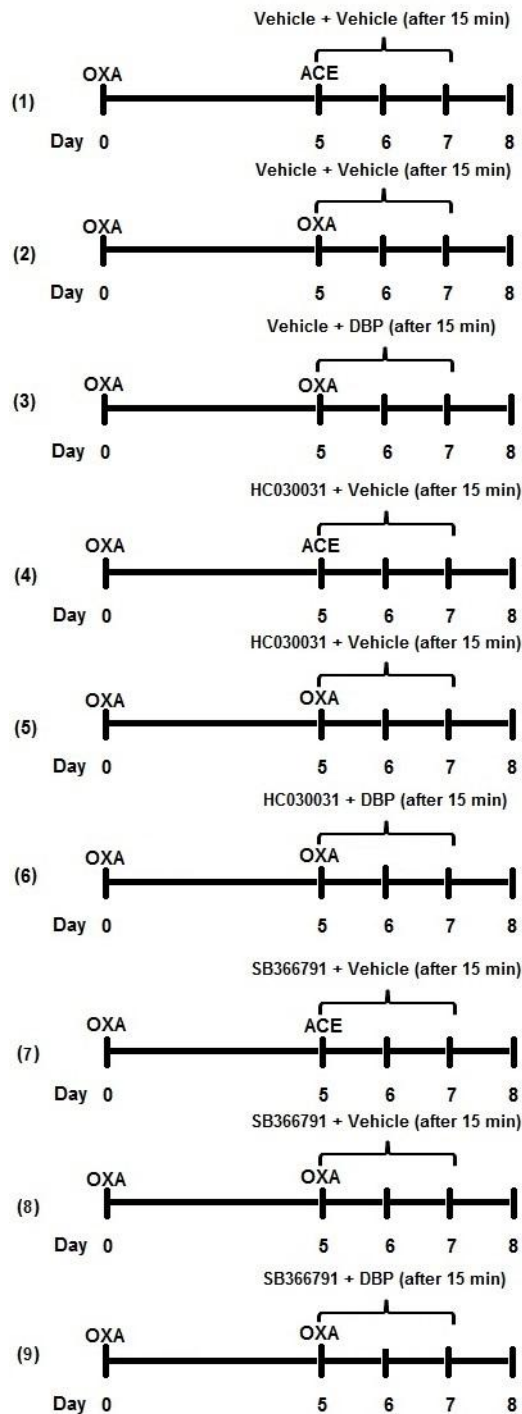


FIGURE 1. EXPERIMENTAL DESIGN. Male BALB/c mice were treated with 30 μ L of Oxazolone (OXA, 0.25% w/v) on their shaven abdomens on day 0 (Sensitization). On day 5, baseline ear thickness was measured followed by challenge with 15 μ L of vehicle (ACE) or Oxazolone (0.5% w/v) on the right ear. After challenge, the ear was treated with 20 μ L of vehicle or 30 nmol/ear of HC030031 or SB366791 followed by treatment with 20 μ L of DBP (0.4 mg/ear) or vehicle. These treatments were performed with 12 hour intervals, totalizing 6 treatments by the end of 72 hours. Ear thickness was measured on days 6, 7 and 8 (24, 48 and 72 hours after challenge). On day 8, the animals were

ethanized and 6 mm circles of ear tissue were collected for future analysis. (1) Negative control group, (2) Positive control group for contact hypersensitivity, (3) DBP treated control group (treated with DBP 0.4 mg/ear), (4) TRPA1 negative control group, (5) TRPA1 positive control group, (6) TRPA1 treated group with DBP 0.4 mg/ear, (7) TRPV1 negative control group, (8) TRPV1 positive control group, (8) TRPV1 treated group with DBP 0.4 mg/ear.

2.4 HISTOLOGICAL ANALYSIS

Ear tissue samples were fixed in ALFAC (10% formaldehyde, 85% ethanol 80%, 5% glacial acetic acid) solution. The ears were later embedded in paraffin, cut at 5 µm sections and stained with hematoxylin–eosin.

The images for analysis were obtained through a motorized microscope Axio Imager Z2 (Carl Zeiss, Jena, Germany), equipped with automated scanner VSlide (Metasystems, Altlussheim, Germany). The scanned images were analyzed with ImageJ software (NIH, USA). Epidermal thickness (considered as the distance from the basal layer to the stratum granulosum) and dermal thickness (considered as the distance from the basal layer of the epidermis to the auricular cartilage) were measured, and the presence of inflammatory cells was quantified. Data are expressed as the mean value of three representative areas of three histological sections of each animal. Epidermal thickness measure and infiltrated cells count were performed in 200x magnification sections, while dermal thickness was measured in 100x magnification sections.

2.5 TISSUE MYELOPEROXIDASE (MPO) ASSAY

MPO is an enzyme used as a marker for the influx of polymorphonuclear leukocytes into inflamed tissues. MPO activity was evaluated according to the method proposed by Bradley *et al.* (1982) and modified by De Young *et al.* (1989). Each ear sample (6 mm circles) was placed in 0.75 mL of 80 mM sodium phosphate buffer (PBS, pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB). Next, the sample was homogenized (45 s at 0 °C) in a motor-driven homogenizer. The homogenate was decanted into a microfuge tube, and 0.75 mL of HTAB in PBS

was added to the tube. The 1.5 mL mixture was centrifuged at $11,200 \times g$ at 4°C for 20 min. The supernatant samples (triplicates of $30\ \mu\text{L}$) were added to 96-well plates. For the assay, $200\ \mu\text{L}$ of a mixture containing $100\ \mu\text{L}$ of $80\ \text{mM}$ PBS (pH 5.4), $85\ \mu\text{L}$ of $0.22\ \text{M}$ PBS (pH 5.4) and $15\ \mu\text{L}$ of 0.017% H_2O_2 were added to the wells. The reaction was started by the addition of $20\ \mu\text{L}$ of $18.4\ \text{mM}$ tetramethylbenzidine HCl (TMB) in dimethylformamide. The mixture was incubated for 3 min at 37°C , and the reaction was subsequently stopped by the addition of $30\ \mu\text{L}$ of $1.46\ \text{M}$ sodium acetate (pH 3.0). Enzyme activity was determined colorimetrically using a plate reader (Bio-Tek Multi-Mode Microplate reader Synergy HT) to measure absorbance at $620\ \text{nm}$, and the results were expressed as mili optical density (mDO) per biopsy.

2.6 TISSUE N-ACETYL- β -D-GLUCOSAMINIDASE (NAG) ASSAY

The activity of NAG was used as a marker for the presence of mononuclear leukocytes in inflamed tissue. According to the method used by Sanchez and Moreno (1999), ear samples (6 mm circles) were treated using the same method described for the MPO assay. The supernatant samples (triplicates of $25\ \mu\text{L}$) were added into 96-well plates. For the assay, $25\ \mu\text{L}$ of p-nitrophenyl-acetamide- μ -d-glucopyranoside ($2.24\ \text{mM}$) and $100\ \mu\text{L}$ of $50\ \text{mM}$ buffer citrate (pH 4.5) per well were used. The mixture was incubated for 60 min at 37°C , and the reaction was stopped by the addition of $100\ \mu\text{L}$ of $200\ \text{nM}$ glycine buffer (pH 10.4). The enzyme activity was determined colorimetrically using a plate reader (Bio-Tek Multi-Mode Microplate reader Synergy HT) to measure absorbance at $405\ \text{nm}$, and enzyme activity was expressed as mDO per biopsy.

2.7 DATA ANALYSIS

The data presented are expressed as mean \pm SEM. Statistical significance was determined by ANOVA followed by Tukey multiple comparison test or Kruskal Wallis followed by Dunn, using the GraphPad Prism software (GraphPad Software,

La Jolla, CA). When necessary, Welch's t test was performed to confirm significant differences. Differences were considered significant for $p \leq 0.05$.

3 RESULTS

3.1 EFFECTS OF DBP ON OXAZOLONE-INDUCED EAR EDEMA IN BALB/c MICE AFTER TREATMENT WITH TRP ANTAGONISTS.

Figure 2 represents the evolution of ear edema 24, 48 and 72 hours after challenge with vehicle or oxazolone followed by treatment with TRPA1 or TRPV1 antagonists and DBP. Oxazolone increased ear edema 24, 48 and 72 hours after challenge when compared with the negative control group. This increase was observed in the group exposed only to oxazolone (treated with vehicle after challenge; positive control group) as well as in the groups exposed to oxazolone and then treated with HC030031 (TRPA1 positive control group) or SB366791 (TRPV1 positive control group). Overall, these TRP antagonists did not change the oxazolone response, except that SB366791 and HC030031 slightly reduced oxazolone-induced edema 24h and 48h after challenge, respectively. No significant differences were observed between these groups 72 hours after challenge.

In animals not treated with TPR antagonists (vehicle only), DBP treatment significantly increased ear edema induced by oxazolone at all time points. However, the adjuvant effect of DBP on ear edema was partially or totally blocked by TRPA1 and TRPV1 antagonists, respectively. Treatment with DBP and HC030031 increased ear edema above the level seen for its respective positive control, but this was significantly lower than the response observed in animals treated with DBP and vehicle at all time points. Treatment with DBP and SB366791 did not increase ear edema in relation to its concurrent positive control. The edema induced in this group was also significantly lower than that seen in animals treated with DBP and vehicle at all time points.

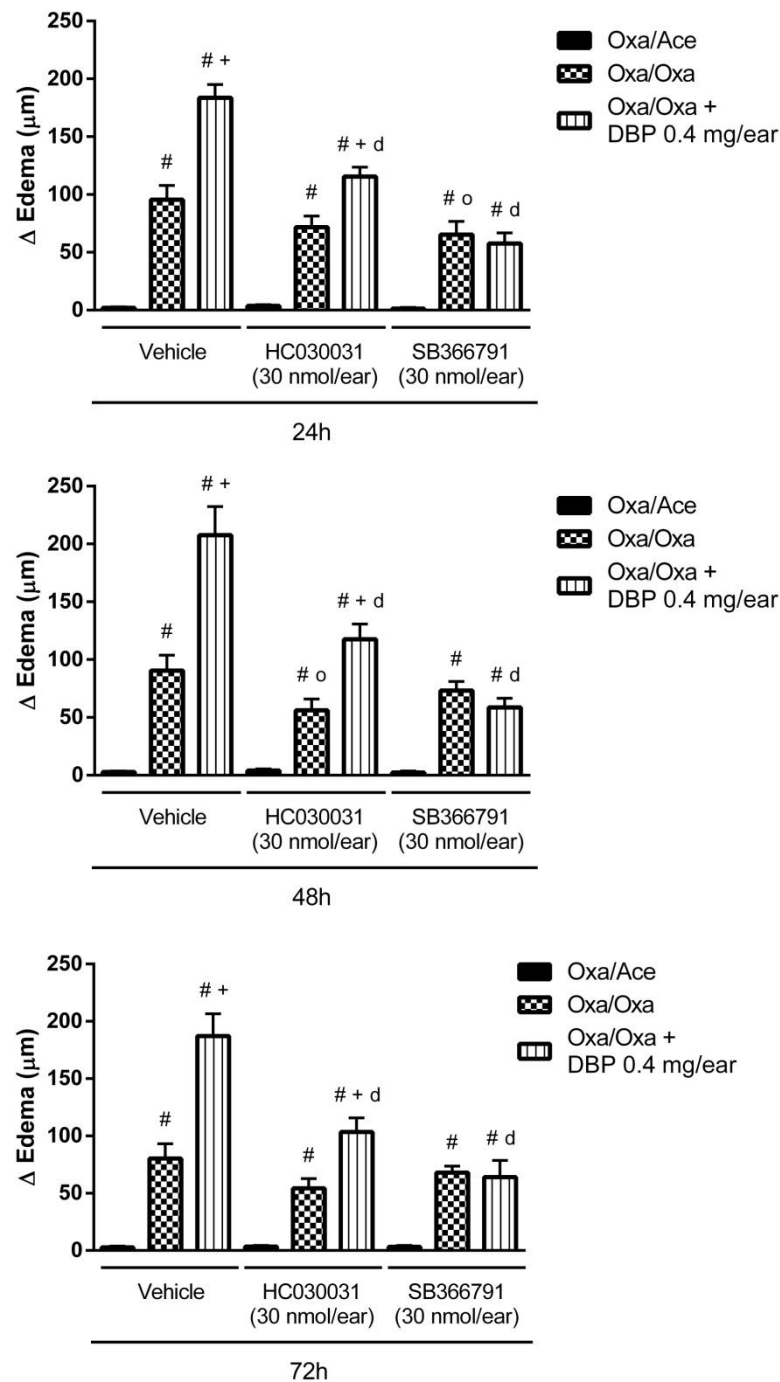


FIGURE 2. EVOLUTION OF EAR EDEMA AFTER CHALLENGE WITH OXAZOLONE AND TREATMENT WITH TRP ANTAGONISTS AND DIBUTYL PHTHALATE. Following abdominal sensitization with oxazolone, the right ear of the animals was challenged with vehicle (Acetone) or oxazolone and then treated with vehicle, HC030031 (TRPA1 antagonist – 30 nmol/ear) or SB366791 (TRPV1 antagonist – 30 nmol/ear), followed by treatment with vehicle or DBP (0.4 mg/ear). Ear thickness was measured 24, 48 and 72 hours after challenge. Sensitization/Elicitation. Oxa/Ace - oxazolone/acetone (negative control group, n=9); Oxa/Oxa - oxazolone/oxazolone (positive control group, n=9); Oxa/Oxa + DBP 0.4 mg/ear - oxazolone/oxazolone + DBP 0.4 mg/ear (n=9). (#) significantly different from the respective negative control group; (+) significantly different from the respective positive control group; (o) significantly different from the positive control group; (d) significantly different from the DBP treated control group. $p < 0,05$, ANOVA followed by Tukey.

3.2 EFFECTS OF TREATMENT WITH ANTAGONISTS AND DBP IN CELL INFILTRATE AND DERMAL AND EPIDERMAL THICKNESS INDUCED BY OXAZOLONE

The topic exposure to oxazolone induced the recruitment of inflammatory cells to the ear tissue as demonstrated in Figures 3B, 3E and 3H. In addition, the association of oxazolone and DBP 0.4 mg/ear appear to have further increased the infiltration of inflammatory cells (FIGURE 3C). However, this further increase was apparently less significant in the TRPA1 and TRPV1 groups (FIGURE 3F and 3I) when compared with their respective positive control groups (FIGURE 3E and 3H), in particular for the group treated with TRPV1 antagonist. These qualitative data were confirmed by the cell count in the dermis, which revealed significant increase in cell number in the DBP treated control (vehicle) group when compared with the positive control group (FIGURE 4). In the group treated with the TRPA1 antagonist (HC030031), DBP-induced increase in cell infiltration was slight and of borderline significance ($p=0.06$) in relation to its concurrent control. On the other hand, the TRPV1 antagonist completely blocked the increase in the number of infiltrated cells induced by DBP. In addition, the number of infiltrated cells in the group treated with TRPV1 antagonist and DBP was significantly lower than that seen in animals treated with vehicle and DBP. There was no significant difference among the three positive control groups.

Both dermal and epidermal thickness were increased in animals sensitized and challenged with oxazolone when compared with their respective negative control groups. In addition, in groups treated with vehicle or TRPA1 antagonist, DBP significantly increased dermal and epidermal thickness in comparison with their respective positive control groups (FIGURE 3 and 4). No difference in both parameters were found among the positive control groups, but both TRPA1 and TRPV1 antagonists significantly reduced DBP-induced increase in dermal and epidermal thickness when compared with animals treated with vehicle and DBP. For HC030031 the reduction in dermal thickness was of borderline significance ($p=0.06$).

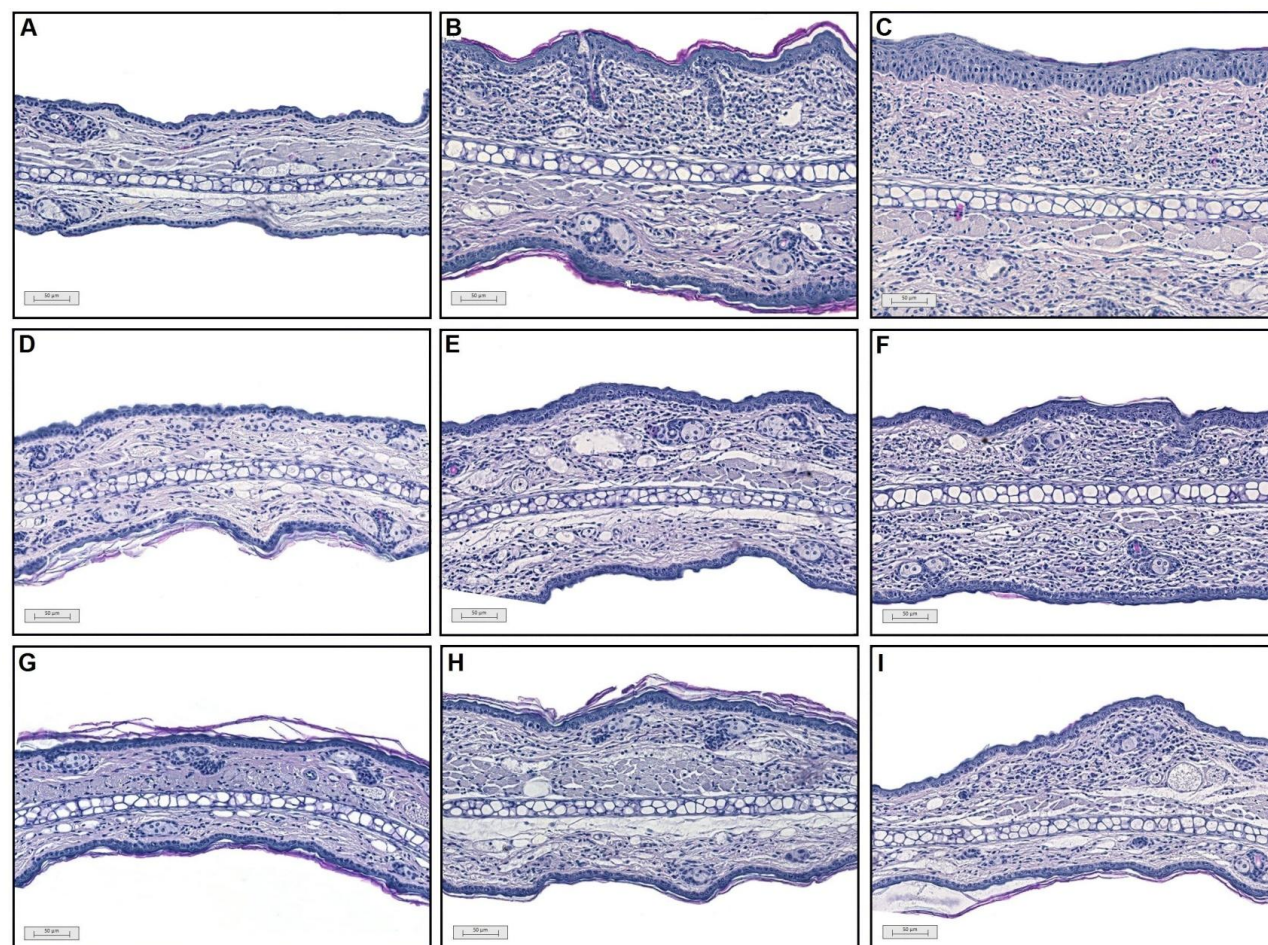


FIGURE 3. PHOTOMICROGRAPHS OF THE RIGHT EAR OF REPRESENTATIVE ANIMALS FROM ALL GROUPS (100X). Following abdominal sensitization with oxazolone, the right ear of the animals was challenged with vehicle or oxazolone and then treated with vehicle or TRP antagonists followed by treatment with vehicle or DBP. Sensitization/Elicitation. (A) oxazolone/acetone - negative control group; (B) oxazolone/oxazolone – positive control group ; (C) oxazolone/oxazolone + DBP 0.4 mg/ear – DBP treated control group; (D) oxazolone/acetone – TRPA1 negative control group; (E) oxazolone/oxazolone – TRPA1 positive control group ; (F) oxazolone/oxazolone + DBP 0.4 mg/ear – TRPA1 treated group; (G) oxazolone/acetone – TRPV1 negative control group; (H) oxazolone/oxazolone – TRPV1 positive control group; (I) oxazolone/oxazolone + DBP 0.4 mg/ear – TRPV1 treated group.

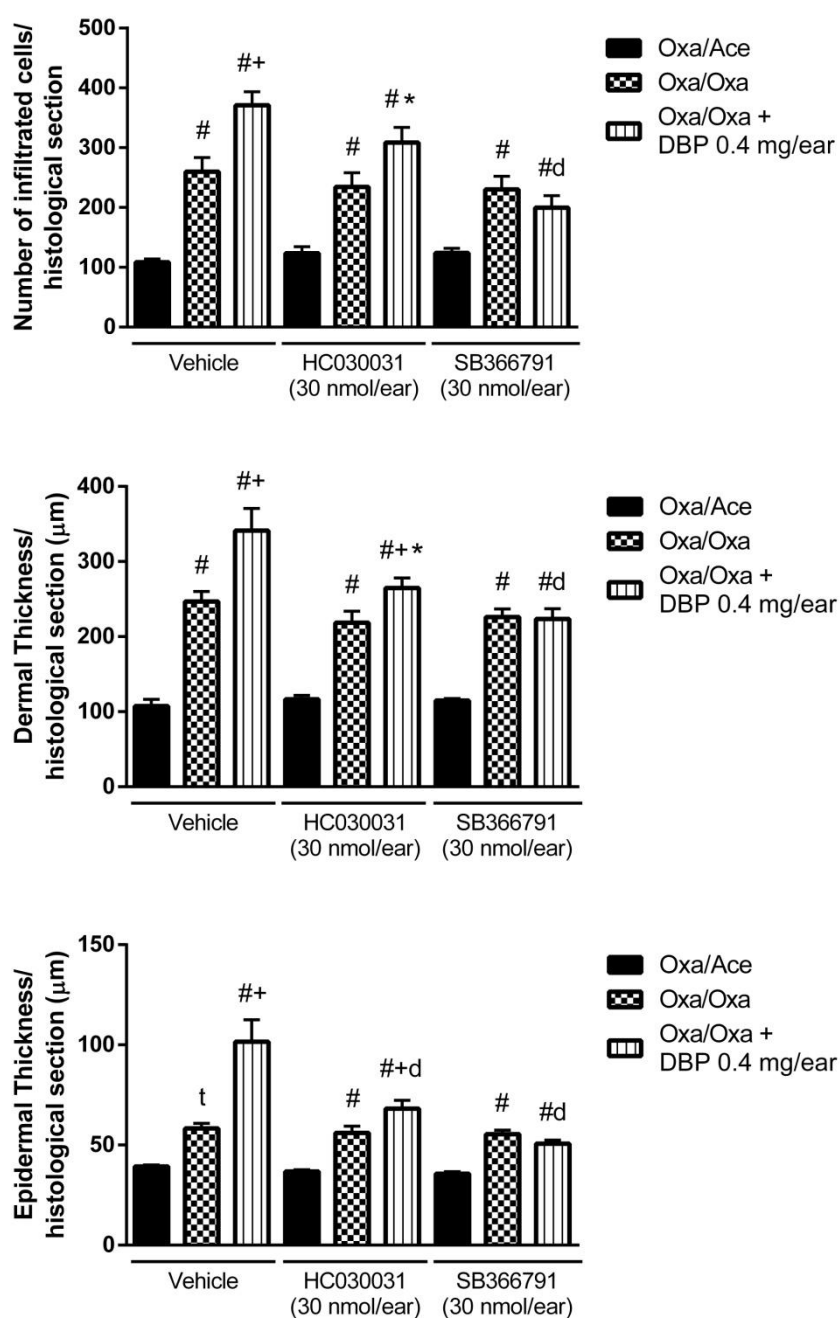


FIGURE 4. HISTOLOGICAL PARAMETERS. Following abdominal sensitization with oxazolone, the right ear of the animals was challenged with vehicle or oxazolone and then treated with vehicle or TRP antagonists followed by treatment with vehicle or DBP. For each animal, histological parameters were evaluated in three representative areas of three histological sections. Sensitization/Elicitation. Oxa/Ace - oxazolone/acetone (n=5); Oxa/Oxa - oxazolone/oxazolone (n=5); Oxa/Oxa + DBP 0.4 mg/ear - oxazolone/oxazolone + DBP 0.4 mg/ear (n=5). (#) significantly different from the respective negative control group; (+) significantly different from the respective positive control group; (d) significantly different from the DBP treated control group; (*) Number of infiltrated cells) p=0.06 when compared to the TRPA1 positive control group; (*) Dermal thickness) p=0.06 when compared with the DBP treated control group. p<0.05, ANOVA followed by Tukey. (t) significantly different from the negative control group. p<0.05, Welch's t test.

3.3 EFFECTS OF EXPOSURE TO OXAZOLONE FOLLOWED BY TREATMENT WITH TRP ANTAGONISTS AND DBP IN MPO AND NAG ACTIVITY

Although there was an apparent increase in MPO activity in the positive control groups when compared with their respective negative control groups, no statistically significant difference was found. These groups were significantly different only from animals that did not receive any treatment (naïve). DBP seemed to have increased MPO activity in rats treated with vehicle, but this effect was also not significant. In the group treated with TRPA1 antagonist and DBP MPO activity was diminished in relation to the group treated with vehicle and DBP. Similarly, the group treated with TRPV1 antagonist and DBP displayed lower MPO activity in relation to the group treated with vehicle and DBP, but this effects was only of borderline significance ($p=0.1$).

NAG activity was increased in the three positive control groups when compared with their respective negative control groups. DBP treatment increased NAG activity above the level seen for the positive control group in animals treated with vehicle. TRPA1 and TRPV1 antagonists significantly blocked the DBP-induced increase in NAG activity. These groups were not different from their respective controls and were significant lower than the levels seen for animals treated with vehicle and DBP.

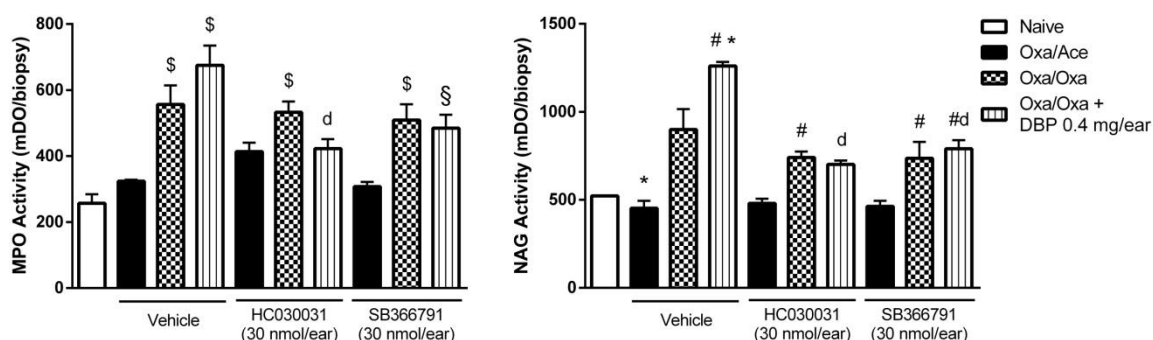


FIGURE 5. EFFECTS OF EXPOSURE TO DBP ASSOCIATED TO OXAZOLONE ON MPO AND NAG ACTIVITY. Following abdominal sensitization with oxazolone, the right ear of the animals was challenged with vehicle or oxazolone and then treated with vehicle or TRP antagonists followed by treatment with vehicle or DBP. Naïve: animals were not exposed to any substance. Sensitization/Elicitation. Oxa/Ace - oxazolone/acetone ($n=4$); Oxa/Oxa - oxazolone/oxazolone ($n=4$); Oxa/Oxa + DBP 0.4 mg/ear - oxazolone/oxazolone + DBP 0.4 mg/ear ($n=4$). (\$) significantly different from naïve group; (#) significantly different from the respective negative control group; (d) significantly different from the DBP treated control, (\$) $p=0.1$ when compared with the DBP treated control group, (*) $p=0.057$ when compared with the positive control group. $p<0.05$, Mann-Whitney test.

4 DISCUSSION

The prevalence of allergic diseases, as atopic dermatitis, asthma and rhinitis has been increasing (SEGAWA; HIRASAWA, 2014), and this could be related to the presence of chemicals with immune adjuvancy potential in the environment. Phthalate esters, which are widely found in the environment, are candidate chemicals with adjuvant effects (LARSEN *et al.*, 2002). Epidemiological data indicate that certain phthalate esters could participate in the development of allergic diseases such as asthma and atopic dermatitis (BORNEHAG *et al.*, 2004; JAAKKOLA; KNIGHT, 2004). Besides, it's been reported that phthalates could enhance the immune response to allergens in animal models of CHS where phthalates are present in the sensitization phase (IMAI *et al.*, 2006; Li *et al.*, 2014; MATSUDA *et al.*, 2010). We have previously demonstrated that DBP aggravates the immune response in an animal model of CHS induced by oxazolone in BALB/c mice when DBP is associated to oxazolone in the elicitation phase of the model. We observed increase in ear edema, polymorphonuclear and mononuclear leukocyte infiltration and epidermal hyperplasia. In addition, we have also investigated the adjuvant effect of DBP in two *in vitro* models of contact hypersensitivity, the NCTC 2544 IL-18 assay (used as surrogate of contact allergen-induced keratinocyte activation) and the THP-1 activation assay (used as marker of dendritic cell activation). We found that DBP potentiates chemical allergen-induced THP-1 activation but no adjuvant effect was observed in the activation of NCTC 2544 cells (LOURENÇO *et al.*, 2015).

It has been reported that phthalates could activate TRPA1 and TRPV1 *in vitro* (SHIBA *et al.*, 2009; JINNO; OHKAWARA; TANAKA-KAGAWA, 2012). In addition, Maruyama *et al.* (2007) showed that desensitization of sensory neurons by local treatment with AITC or CAP suppressed skin sensitization to FITC in the presence of DBP in a mouse model. This was associated with reduced maturation and trafficking of FITC-presenting dendritic cells. Besides, a CGRP antagonist also suppressed the sensitization to FITC, suggesting a possible involvement of sensory neurons in sensitization. Shiba *et al.* (2012) also reported that pretreatment with TRPA1 antagonist HC030031 upon skin sensitization to FITC suppressed the enhancing effect of DBP on sensitization.

In the present study we demonstrated that local treatment with TRPA1 (HC030031) and TRPV1 antagonists (SB366791) partially or completely suppressed, respectively, the adjuvant effect of DBP in a BALB/c model of CHS induced by the allergen oxazolone. These TRP antagonists were able to reverse DBP's adjuvant effect on ear edema, leukocyte infiltration and epidermal hyperplasia in oxazolone-challenged animals. Even though others have shown the reduction in ear swelling enhanced by phthalates after desensitization of sensory neurons or using TRP or neuropeptide antagonists (MARUYAMA *et al.*, 2007; SHIBA *et al.*, 2012), this was showed when DBP was associated in both phases of the CHS model. Our results show that even after the sensitization has already been established, DBP can still exert adjuvant effect and enhance the inflammatory response, modulating the elicitation phase of CHS induced by oxazolone. More importantly, treatment with TRPA1 and TRPV1 antagonists after challenge with oxazolone was enough to partially or completely prevent the adjuvant effect of DBP in oxazolone-induced CHS, corroborating the hypothesis that TRPs are molecular targets that mediate inflammatory responses induced by phthalates.

It is important to highlight that overall no difference was found among the positive control groups, i.e., oxazolone response was not modified by treatment with TRP antagonists. Banvolgyi *et al.* (2005) showed that TRPV1-deficient mice presented enhanced response in CHS induced by oxazolone. On the other hand, Liu *et al.* (2013) demonstrated that treatment with TRPA1 antagonist HC030031, prior challenge, inhibited skin edema, keratinocyte hyperplasia, nerve growth, leukocyte infiltration, and antihistamine-resistant scratching behavior in mice exposed to oxazolone. In addition, it's been shown that CGRP applied topically at the site of contact with the allergen, during both sensitization and elicitation phase, enhanced the sensitization in oxazolone-induced CHS (GUTWALD; GOEBELER; SORG, 1991). Different from previous studies, that intended to investigate the effects of TRP in the sensitization in oxazolone-induced CHS, in the present study we have chosen to expose the animals to TRP antagonists only after challenge with oxazolone, aiming to investigate the modulation of these antagonists in the adjuvant effect of DBP in oxazolone-induced CHS; therefore no modulation on oxazolone effect was observed.

In this study, both TRPV1 and TRPA1 antagonists were able to reverse the adjuvant effects of DBP. Animals treated with TRPA1 antagonist and DBP presented reduced ear edema and dermal and epidermal thickness when compared with the

DBP control group, although these parameters were still higher in this group in comparison with TRPA1 positive control group. On the contrary, TRPV1 antagonist reversed all of the parameters analyzed in animals treated with TRPV1 antagonist and DBP, in relation to TRPV1 positive control group. We have previously demonstrated that DBP is capable of potentiating allergen-induced activation of THP-1 cells *in vitro*, which are cells used as surrogates for dendritic cell-activation (LOURENÇO *et al.*, 2015). In addition, it has been reported that both TRPV1 and TRPA1 are expressed on dendritic cells (DC) and that the activation of TRPV1 in DC could induce their maturation and enhance their antigen presenting activity (BASU; SRIVASTAVA, 2005). On the other hand, in our previous *in vitro* study, DBP did not present any adjuvant effect in the allergen-induced activation of keratinocytes (NCTC 2544 cells). However, others have shown that DBP increased the production of thymic stromal lymphopoietin (TSLP) in FITC-induced contact hypersensitivity in mice (SHIGENO *et al.*, 2009; LARSON *et al.*, 2010), but the mechanism of TSLP production upon exposure to DBP was not evaluated in those studies. TSLP is an IL-7-related cytokine, produced by epithelial cells, like keratinocytes, that has been linked to atopic dermatitis and asthma (LEYVA-CASTILLO *et al.*, 2013). It has been also demonstrated that TRPA1 are expressed in human keratinocytes (ATOYAN; SHANDER; BOTCHKAREVA, 2009), and Shiba *et al.* (2012) suggested that it is possible that TRPA1 activation on keratinocytes could trigger the production of TSLP, and that this could be part of the mechanism of enhanced skin-sensitization to FITC induced by phthalates. DBP could activate TRPV1 and TRPA1 directly in dendritic cells enhancing their maturation and antigen presenting activity, besides activating these receptors in keratinocytes and also in sensory neurons triggering the release of neuropeptides such as CGRP and SP, that have been shown to participate in CHS response (MARUYAMA *et al.*, 2007; MIKAMI *et al.*, 2011; LIU *et al.*, 2013).

In conclusion, our data suggest that both TRPA1 and TRPV1 activation could participate in the adjuvant effect of DBP in the elicitation phase of oxazolone-induced CHS model. Our data contribute to further understanding of the molecular mechanisms involved in the adjuvant effect of phthalates.

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5.4.1 Material Complementar

5.4.1.1 Highlights

TRPA1 antagonist reduces adjuvant effect of dibutyl phthalate in oxazolone-induced contact hypersensitivity.

TRPV1 antagonist completely reverses adjuvant effect of dibutyl phthalate in oxazolone-induced contact hypersensitivity.

5.4.1.2 Graphic Abstract

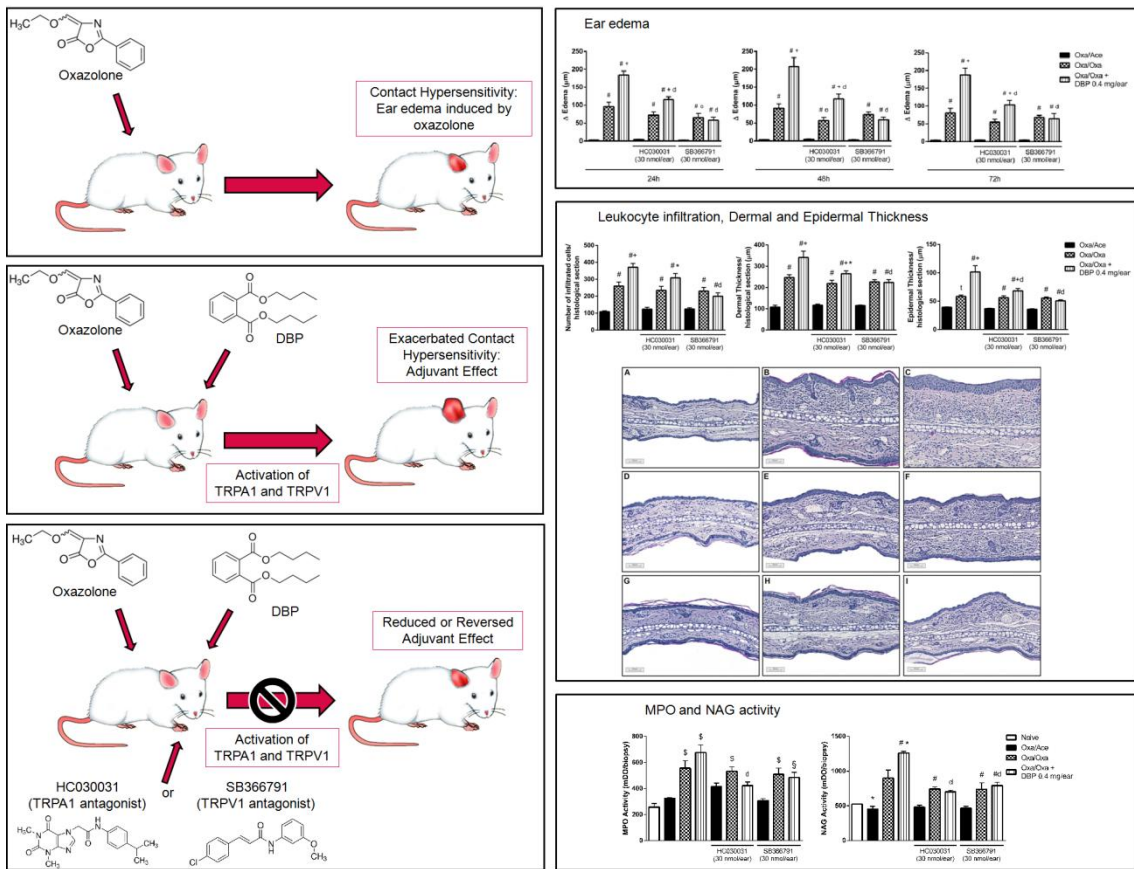


FIGURA 11. GRAPHIC ABSTRACT.

6 DISCUSSÃO GERAL

Como os seres humanos são constantemente expostos a ftalatos, é importante elucidar o potencial imunotóxico dos mesmos. Estudos toxicológicos têm demonstrado que ésteres de ftalatos, como o DBP, não possuem um potencial significativo de causarem sensibilização da pele, pois não são capazes de se ligarem a proteínas e formar um complexo hapteno-proteína, pré-requisito para a estimulação alérgica por uma substância química (KIMBER; DEARMAN, 2010). De acordo com dados epidemiológicos, ésteres de ftalatos não tem o potencial de causar irritação ou sensibilização da pele (GEIER *et al.*, 2004; MEDEIROS; DEVLIN; KELLER, 1999). No entanto, mesmo que evidências apoiem o potencial sensibilizante negativo dos ftalatos, tem sido sugerido que certos ftalatos poderiam atuar como adjuvantes, acelerando, prolongando ou exacerbando as respostas imunes.

A prevalência de doenças alérgicas, como dermatites, asma e rinite, tem aumentado nas últimas décadas (SEGAWA; HIRASAWA, 2014), e isso poderia estar relacionado não somente com o aumento de substâncias sensibilizantes, como também com a presença de substâncias químicas com potencial adjuvante no ambiente. Os ésteres de ftalatos, amplamente encontrados no ambiente, são possíveis candidatos com potencial adjuvante (LARSEN *et al.*, 2002). Tem sido proposto que os ftalatos possam estar associados com facilitação no desenvolvimento de doenças alérgicas como asma e dermatite (BORNEHAG *et al.*, 2004; JAAKKOLA; KNIGHT, 2004), além de ter sido demonstrado que certos ftalatos são capazes de aumentar a resposta a alérgenos em modelos animais de dermatite de contato alérgica (IMAI *et al.*, 2006; LI *et al.*, 2014). Apesar de estudos anteriores *in vivo* terem demonstrado que certos ftalatos são capazes de agravar a resposta e/ou doenças alérgicas, os mecanismos celulares e moleculares envolvidos permanecem obscuros.

A identificação de substâncias sensibilizantes é feita principalmente pelo uso de modelos animais de inflamação cutânea, pois os mesmos mimetizam alguns eventos observados em dermatites desenvolvidas por humanos (VANE; BOTTING, 1998; BOUCLIER *et al.*, 1990). O modelo de edema de orelha induzido pela

oxazolona (FUJII *et al.*, 2002) tem sido bastante utilizado com esse propósito, pois se assemelha à hipersensibilidade de contato em humanos. No entanto, novos ensaios *in vitro* têm sido desenvolvidos com o propósito de encontrar marcadores biológicos capazes de discriminar substâncias sensibilizantes as quais os humanos estejam expostos. Nesse contexto, o ensaio NCTC 2544 IL-18 e o teste de ativação de células THP-1 (CORSINI *et al.*, 2009; GALBIATI *et al.*, 2011; CORSINI *et al.*, 2013a; MITJANS *et al.*, 2008) têm sido bastante utilizados com esse propósito.

O presente estudo investigou os efeitos do DBP nos modelos *in vitro* e *in vivo* de hipersensibilidade de contato acima citados. Os resultados obtidos indicam que o DBP é capaz de agravar a resposta imune e inflamatória envolvida nestes modelos. Neste estudo, observamos que o DBP foi capaz de potencializar a ativação de células THP-1 induzida por alérgenos, aumentando assim a expressão do marcador de superfície celular CD86 e a liberação de interleucina-8. Além disso, demonstramos que o DBP foi capaz de agravar a resposta inflamatória no modelo de edema de orelha induzido por oxazolona, quando associado a este hapteno durante a fase de elicitação do modelo, o que foi demonstrado por aumento em edema, recrutamento de leucócitos e hiperplasia epidérmica. Também investigamos possíveis mecanismos do efeito adjuvante exercido pelo DBP. Identificamos *in vitro* uma participação da produção de espécies reativas de oxigênio na ativação de células THP-1. *In vivo*, observamos a participação da ativação dos receptores TRPA1 e TRPV1 no efeito do DBP. Os dados obtidos neste estudo mostram que os ftalatos podem estar agindo como adjuvantes não somente na fase de sensibilização de processos alérgicos, como também na fase de elicitação, e que esse efeito pode estar mais relacionado com o aumento da ativação de células dendríticas apresentadoras de antígenos. Além disso, nossos resultados contribuem para um maior entendimento dos mecanismos envolvidos no efeito adjuvante dos ftalatos em modelos de hipersensibilidade de contato.

Os resultados encontrados neste estudo apoiam dados epidemiológicos e experimentais que sugerem que o DBP não apresenta potencial significativo de causar sensibilização ou irritação da pele (KIMBER; DEARMAN, 2010; GEIER *et al.*, 2004; MEDEIROS; DEVLIN; KELLER, 1999). Observamos que o DBP não foi capaz de induzir edema, recrutamento celular ou hiperplasia epidérmica no modelo *in vivo*, além de não induzir a ativação de células THP-1 ou NCTC nos ensaios *in vitro* (FIGURAS 07, 08 e 10).

A partir da análise de edema e da contagem de células infiltradas na derme, observou-se que o DBP nas doses mais altas (0,4 e 4 mg/orelha) foi capaz de exacerbar o desenvolvimento do edema de orelha induzido por oxazolona em camundongos, além de aumentar o recrutamento de leucócitos para a derme. Somado a isso, a proliferação de células na epiderme é característica da dermatite de contato causada pela oxazolona (FUJII *et al.*, 2002). Novamente, DBP 0,4 e 4 mg/orelha induziram aumento na hiperplasia epidérmica induzida por oxazolona.

O aumento do edema de orelha (IMAI *et al.*, 2006; SHIBA *et al.*, 2009; MATSUDA *et al.*, 2010; LI *et al.*, 2014) e da infiltração celular (TAKANO *et al.*, 2006; LI *et al.*, 2014) demonstrados nesse experimento já foi relatado por outros autores, embora utilizando doses maiores de ftalatos. Imai e colaboradores (2006) demonstraram que, não só o DBP, como outros ftalatos foram capazes de exacerbar o edema de orelha induzido pelo isotiocianato de fluoresceína. Takano e colaboradores (2006) demonstraram o aumento do edema de orelha e também da infiltração celular no sítio inflamatório em um modelo de dermatite atópica no qual os animais foram expostos ao DEHP. Esses mesmos autores demonstraram ainda que a exposição materna ao ftalato DEHP durante a lactação resultou em exacerbação do edema de orelha e da migração celular na prole das fêmeas expostas, mostrando que talvez os efeitos dos ftalatos após exposição durante períodos críticos para o desenvolvimento, como a gestação e a lactação, não se resumam a alterações no sistema reprodutivo masculino (YANAGISAWA *et al.*, 2008). Já Li e colaboradores (2014) mostraram que a exposição dérmica prolongada (40 dias) ao DBP em doses de 4,0 e 40 mg/kg/dia foi capaz de aumentar o edema de orelha e a infiltração de leucócitos na derme. Nossos dados apoiam o potencial adjuvante do DBP em modelos animais de hipersensibilidade de contato, mas aqui reportamos um efeito adjuvante após um curto período de exposição dérmica a baixas doses de DBP. Adicionalmente, nossos resultados indicam que o DBP pode atuar como adjuvante mesmo quando a sensibilização ao alérgeno já se encontra instalada, modulando a fase de elicitação da HSC induzida por oxazolona. Estudos anteriores têm observado este efeito em modelos animais de HSC associando ftalatos tanto na fase de sensibilização quanto na fase de elicitação, ou com altas doses de ftalatos, exposição por longos períodos de tempo ou em diferentes vias de exposição, em alguns casos com injeções subcutâneas ou intraperitoneais, que não são vias pelas quais os humanos encontrariam normalmente os ftalatos no ambiente.

O presente estudo se propôs a investigar alguns aspectos relacionados ao mecanismo do efeito adjuvante do DBP em processos alérgico-inflamatórios. Para isso foram utilizados dois ensaios *in vitro* baseados na ativação de dois tipos celulares que desempenham papel importante no desenvolvimento da hipersensibilidade de contato, os queratinócitos e as células dendríticas. Em particular, a produção de IL-18 em células NCTC 2544 é representativa de ativação de queratinócitos induzida por alérgenos, enquanto a expressão aumentada de CD86 e produção de IL-8 em células THP-1 foram usadas como marcadores de ativação de células dendríticas. Os resultados obtidos sugerem que o alvo do efeito adjuvante do DBP são as células dendríticas, mas não os queratinócitos, já que o DBP não foi capaz de aumentar a produção de IL-18 induzida por p-Fenilenodiamina (PPD) em células NCTC.

Mitjans *et al.* (2008) demonstraram que alérgenos químicos induzem uma liberação dose dependente de interleucina 8, assim como a expressão do marcador de superfície CD86 na linhagem celular monocítica humana THP-1, propondo isto como um ensaio *in vitro* para screening de potenciais alérgenos de contato. Neste trabalho, o aumento na expressão de CD86 e na liberação de IL-8 foram investigados após exposição de células THP-1 a dois alérgenos de contato, citral e imidazolidinil ureia, e suas associações com DBP. De acordo com o teste do linfonodo local (LLNA), citral é classificado como um sensibilizante moderado com um valor EC₃ de 9,3%, enquanto o imidazolidinil ureia é classificado como um sensibilizante fraco com um valor EC₃ de 24%. Ambos alérgenos induziram um aumento significativo na expressão de CD86 e liberação de IL-8. Quando células THP-1 foram expostas a Cit ou IMZ associados ao DBP, as células exibiram maior expressão de CD86 e maior liberação de IL-8, quando comparadas as células expostas a somente os alérgenos. Além disso, associação entre DBP e oxazolona também revelou maior expressão de CD86 em relação ao grupo exposto a somente oxazolona. Estes resultados indicam um efeito adjuvante do DBP sobre a ativação de células THP-1. Apesar de vários estudos terem sido realizados para avaliar os efeitos dos ftalatos no funcionamento celular, pouco se encontra na literatura sobre o efeito adjuvante destas substâncias *in vitro*. No entanto, uma grande quantidade de estudos sobre o efeito adjuvante de ftalatos *in vivo* tem sido realizada. Diversos autores já demonstraram que diferentes ftalatos podem agir como adjuvantes em hipersensibilidade de contato e inflamação das vias aéreas, elevando a expressão

de marcadores de superfície e citocinas relacionadas após exposição a associações entre alérgenos e ftalatos em diferentes protocolos (IMAI *et al.*, 2006; KOIKE *et al.*, 2010; MATSUDA *et al.*, 2010; LI *et al.*, 2014). Corroborando nossos dados, TANAKA *et al.* (2012) demonstraram que o DEHP agravou a inflamação peritoneal induzida por ovalbumina caracterizada por aumento na infiltração de macrófagos e neutrófilos e por produção e liberação aumentada de citocinas como quimiocina derivada dos queratinócitos (KC) na cavidade peritoneal. KC é um homólogo murino de IL-8 (HOL; WILHELMSSEN; HARALDSEN, 2010). Além disso, Takano *et al.* (2006) mostraram que a exposição a DEHP na presença de alérgeno aumentou a expressão de MIP-1 α comparado a exposição a somente o alérgeno. MIP-1 α funciona como quimiotático de neutrófilos assim como IL-8 (HAMMOND *et al.*, 1995; MENTEN; WUYTS; VAN DAMME, 2002).

A oxazolona é muito utilizada como modelo de hipersensibilidade por contato, por promover as fases de sensibilização e elicitação. Porém, a grande maioria dos estudos avalia os efeitos causados pela aplicação dessas substâncias nos linfonodos e na epiderme, sendo que pouco é discutido sobre as propriedades inflamatórias na derme (ZHANG; THINKLE, 2000). A avaliação da atividade das enzimas NAG e MPO foi realizada para indicar a presença de leucócitos mononucleares e polimorfonucleares, respectivamente (SANCHEZ; MORENO, 1999; BRADLEY *et al.*, 1982), e com isso, caracterizar o possível perfil das células infiltradas na derme. A partir dessas dosagens, sugere-se que tanto leucócitos polimorfonucleares (neutrófilos), quanto mononucleares (macrófagos) estão presentes na resposta inflamatória causada pela oxazolona. Esse resultado está de acordo com o encontrado por Wiedow e colaboradores (1992), que quantificaram proteases de macrófagos e neutrófilos na derme, sendo que estas estavam aumentadas na dermatite de contato alérgica. Sugere-se também que há uma proporção maior de neutrófilos em relação aos leucócitos mononucleares, uma vez que o efeito adjuvante ocasionado pelo DBP aumentou em maior proporção a atividade da enzima MPO em relação à NAG. Os neutrófilos estão envolvidos nas fases de sensibilização e elicitação da HSC, e são uma das primeiras células a serem recrutadas após o desafio de camundongos sensibilizados (DILULIO *et al.*, 1999). Além disso, Zhang e Tinkle (2000) encontraram neutrófilos em maior quantidade na derme em camundongos tratados com oxazolona e permanecem dominantes até 72 horas após o tratamento. Assim, nossos resultados estão de

acordo com outros dados científicos, sendo que o DBP parece exacerbar a resposta inflamatória da oxazolona através do aumento da migração dos neutrófilos, principalmente. Nossos dados *in vitro* concordam com os dados *in vivo* sobre uma maior participação de neutrófilos associados à resposta inflamatória. O aumento da ativação de células dendríticas demonstrada *in vitro* leva a uma maior liberação de IL-8, um potente atrativo de neutrófilos (BARKER *et al.*, 1991). Como mencionado anteriormente, já foi demonstrado em modelos animais um aumento de citocinas como quimiocina derivada dos queratinócitos (KC) (TANAKA *et al.*, 2012), um homólogo murino de IL-8 (HOL; WILHELMSEN; HARALDSEN, 2010), e de MIP-1 α (TAKANO *et al.*, 2006) que também atua como quimiotático de neutrófilos (HAMMOND *et al.*, 1995; MENTEN; WUYTS; VAN DAMME, 2002). Portanto, DBP poderia estar potencializando a ativação de células dendríticas *in vivo* e isso poderia refletir em maior recrutamento de neutrófilos como parte do mecanismo da ação adjuvante do DBP.

Algumas evidências indicam que doenças inflamatórias e alérgicas de pele poderiam ser mediadas por estresse oxidativo (OKAYAMA, 2005; BYAMBA *et al.*, 2010). Mizuashi *et al.* (2005) hipotetizaram que substâncias sensibilizantes induzem estresse oxidativo em células dendríticas, o que subsequentemente estimula p38 MAPK. Seus resultados demonstraram que sensibilizantes reduziram a forma oxidada (GSSG) versus a forma reduzida (GSH) de glutathiona celular (razão GSH/GSSG) em células THP-1 e levaram a fosforilação de p38 MAPK. De acordo com Mitjans *et al.* (2008), a ativação de p38 MAPK por sensibilizantes desempenha um papel crucial na ativação da liberação de IL-8. No presente estudo, juntamente com o aumento na liberação de IL-8, foi demonstrado um pequeno aumento na geração de espécies reativas de oxigênio induzido por DBP, e, mais importante, um aumento significativo no estresse oxidativo induzido por DBP em associação ao citral. Outros já demonstraram que alguns ftalatos podem induzir estresse oxidativo. Tetz *et al.* (2013) reportou um aumento da produção de ROS induzido por MEHP em células placentárias humanas. DEHP também aumentou a geração de ROS em testículos, levando a apoptose de espermátócitos (KASAHARA *et al.*, 2002). Ainda, You *et al.* (2014) mostraram níveis de ROS aumentados significativamente em animais sensibilizados com ovalbumina e tratados com DEHP quando comparados aos níveis de ROS dos animais apenas sensibilizados. Esses autores sugerem que o estresse oxidativo desempenhe um papel crítico no efeito adjuvante do DEHP.

In vivo, demonstramos uma queda em níveis de GSH induzida por oxazolona no grupo controle positivo e também no controle negativo, que foi exposto a oxazolona somente na fase de sensibilização. Esta redução foi acompanhada de aumento nos níveis de LOOH no grupo controle positivo. O estresse oxidativo induzido pela oxazolona em modelo de hipersensibilidade de contato já foi reportado (BRUNYÁNSZKI *et al.*, 2010). Também demonstramos que a sensibilização e desafio com DBP 0.4 mg reduziu os níveis de GSH, mas não foi observado aumento em LOOH neste grupo. Já que muitas vias estão envolvidas na neutralização de EROs, é difícil especular a razão pela qual DBP induziu uma diminuição em GSH sem aumentar LOOH. O que é interessante, é que a mesma dose de DBP (0.4 mg) reverteu parcialmente a redução em GSH e aumento em LOOH induzida por oxazolona, enquanto DBP 0.04 mg reverteu completamente os mesmos parâmetros, e o grupo tratado com a maior dose de DBP (4mg) não diferiu do grupo controle positivo. Nossos resultados parecem indicar que o estresse oxidativo não participa do efeito adjuvante do DBP no modelo animal avaliado com as doses escolhidas. Chen *et al.* (2015) reportou um fraco efeito adjuvante do DBP em modelo murino de asma e observou que esse efeito não foi mediado por um mecanismo de estresse oxidativo. Apesar de termos reportado que DBP foi capaz de induzir produção de EROs *in vitro* e aumentar essa produção quando induzida por um alérgeno, o tempo no qual foi avaliado esse parâmetro foi diferente da análise *in vivo*. Enquanto *in vitro* a produção de EROs foi avaliada imediatamente após o tratamento das células com DBP associado ao alérgeno, *in vivo* a avaliação dos níveis de GSH e LOOH foi feita três dias após a exposição ao ftalato e à oxazolona, assim, o papel da resposta inflamatória induzida tanto pela oxazolona quanto pelo DBP na produção de EROs deve ser considerado. Além disso, ftalatos parecem apresentar efeitos contrários em estudos sobre seu potencial adjuvante quando são usadas doses altas ou baixas e diferentes tempos e vias de exposição (KIMBER; DEARMAN, 2010), portanto, diferentes doses de ftalatos poderiam influenciar o balanço redox celular de maneiras diferentes.

Além do possível papel do estresse oxidativo no desenvolvimento da hipersensibilidade de contato, avaliamos qual seria a participação da ativação dos receptores TRPA1 e TRPV1 no efeito adjuvante do DBP no modelo de edema de orelha induzido por oxazolona. Estudos já demonstraram que os ftalatos são capazes de ativar tanto TRPA1 quanto TRPV1 *in vitro* (SHIBA *et al.*, 2009; JINNO;

OHKAWARA; TANAKA-KAGAWA, 2012). Além disso, já foi reportada a participação desses receptores em modelos animais de hipersensibilidade de contato. Maruyama *et al.* (2007) mostraram que a dessensibilização de neurônios sensoriais através do tratamento tópico com alil isotiocianato ou capsaicina suprimiram a sensibilização da derme induzida por isotiocianato de fluoresceína (FITC) na presença de DBP em um modelo animal, o que foi associado a redução da maturação e migração de células dendríticas. Adicionalmente, um antagonista do neuropeptídeo CGRP também foi capaz de suprimir a sensibilização ao FITC, apoiando o possível envolvimento dos neurônios sensoriais na sensibilização. Shiba *et al.* (2012) também demonstraram que o pré tratamento com o antagonista TRPA1 HC030031 durante a sensibilização ao FITC revogou o efeito adjuvante do DBP na sensibilização.

Neste estudo demonstramos que o tratamento tópico com o antagonista TRPA1 HC030031 e com o antagonista TRPV1 SB366791 após o tratamento com oxazolona, mas antes da exposição ao DBP, suprimiram parcial ou completamente, respectivamente, o efeito adjuvante do DBP, como pudemos observar através da redução do edema de orelha, da infiltração de leucócitos e da hiperplasia epidérmica nos grupos tratados com os antagonistas antes de serem expostos ao DBP. Apesar de outros autores já terem reportado uma redução no edema de orelha aumentado por ftalatos após a dessensibilização de neurônios sensoriais usando antagonistas de TRPs ou de neuropeptídeos (MARUYAMA *et al.*, 2007; SHIBA *et al.*, 2012), tais efeitos foram demonstrados após associação do DBP nas duas fases do modelo de HSC, e do tratamento com os antagonistas ser realizado antes da fase de sensibilização. Nossos resultados mostram que mesmo após a sensibilização ao alérgeno já ter sido estabelecida, o DBP ainda pode exercer efeito adjuvante e aumentar a resposta inflamatória através da modulação da elicitação da HSC induzida por oxazolona, e, ainda, que o tratamento com os antagonistas dos receptores TRPA1 ou TRPV1 após o desafio com oxazolona foi suficiente para prevenir parcial ou completamente o efeito adjuvante do DBP na HSC induzida por oxazolona.

É importante ressaltar que não houve diferença significativa entre os grupos controles positivos. Banvolgyi *et al.* (2005) mostrou que camundongos que não expressavam TRPV1 apresentaram uma resposta aumentada durante a HSC induzida por oxazolona. Por outro lado, Liu *et al.* (2013) demonstraram que o tratamento com o antagonista TRPA1 HC030031, antes do desafio, inibiram o

edema, a hiperplasia epidérmica, a infiltração leucocitária e o comportamento de coceira resiste a anti-histamínicos em camundongos expostos a oxazolona. Diferentemente desses estudos, que pretendiam investigar os efeitos dos antagonistas TRPs sobre a sensibilização induzida pela oxazolona, no presente estudo optamos por expor os animais aos antagonistas TRPs apenas após o desafio com oxazolona, procurando investigar somente a modulação desses antagonistas sobre o efeito adjuvante do DBP na HSC induzida por oxazolona, portanto, nenhuma modulação no efeito sensibilizante da oxazolona foi observada.

No presente estudo, ambos antagonistas TRPA1 e TRPV1 foram capazes de reduzir os efeitos adjuvantes do DBP. Animais tratados com antagonista TRPA1 e DBP apresentaram redução no edema de orelha e na espessura da epiderme e da derme, quando comparados aos animais do grupo controle DBP, apesar de estes parâmetros ainda continuarem mais altos neste grupo quando comparado ao seu respectivo controle positivo. Por outro lado, o antagonista TRPV1 foi capaz de reverter completamente os parâmetros analisados, em animais tratados com o antagonista TRPV1 e DBP, aos mesmos níveis do respectivo grupo controle positivo. Demonstramos anteriormente que o DBP é capaz de potencializar a ativação de células THP-1 induzida por alérgenos *in vitro*, modelo *in vitro* usado como substituto de ativação de células dendríticas (LOURENÇO *et al.*, 2015). Além disso, já foi reportado que TRPV1 é expresso em células dendríticas e queratinócitos, e que a ativação de TRPV1 em células dendríticas poderia induzir a maturação e aumentar o potencial de apresentação de antígenos das mesmas (BASU; SRIVASTAVA, 2005). Por outro lado, em nosso estudo *in vitro*, DBP não apresentou efeito adjuvante sobre ativação de queratinócitos (NCTC 2544) induzida por alérgeno. No entanto, outros estudos demonstraram que DBP aumentou a produção da linfopietina estromal tímica (TSLP) durante a hipersensibilidade de contato induzida por FITC em camundongos (SHIGENO *et al.*, 2009; LARSON *et al.*, 2010), mas o mecanismo de produção de TSLP após exposição ao DBP não foi avaliado nesses estudos. TSLP é uma citotina relacionada a IL-7, produzida por células epiteliais, como queratinócitos, que tem sido relacionada com dermatite atópica e asma (LEYVA-CASTILLO *et al.*, 2013). Também foi demonstrado que TRPA1 é expresso em queratinócitos humanos (ATOYAN; SHANDER; BOTCHKAREVA, 2009), e Shiba *et al.* (2012) sugeriram que é possível que a ativação de TRPA1 em queratinócitos poderia induzir a produção de TSLP, e que

isso poderia ser parte do mecanismo de aumento da sensibilização ao FITC induzida por ftalatos. DBP poderia ativar TRPV1 e TRPA1 diretamente em células dendríticas aumentando sua maturação e atividade apresentadora de antígenos além de ativar os receptores em queratinócitos e também em neurônios sensoriais levando a liberação de neuropeptídeos como CGRP e SP, cuja participação na resposta a HSC já foi demonstrada (MARUYAMA *et al.*, 2007; MIKAMI *et al.*, 2011; LIU *et al.*, 2013).

7 CONCLUSÕES

Os dados obtidos no presente estudo demonstram um efeito adjuvante provocado pelo DBP não somente *in vivo* como também *in vitro* quando associado a diferentes substâncias sensibilizantes. Koike *et al.* (2009) e Ito *et al.* (2012), baseados em seus estudos *in vivo* e *in vitro*, sugeriram que o DEHP acelere a dermatite em roedores através da ativação imprópria de células dendríticas. Koike *et al.* (2010) propôs que DINP poderia ativar as células dendríticas em sítios inflamatórios através do aumento da produção de linfopoietina estromal tímica (TSLP). Imai *et al.* (2006) sugeriram que DBP e DPP facilitem o tráfego das células de Langerhans na pele em hipersensibilidade de contato induzida por isotiocianato de fluoresceína. Juntos, nossos resultados corroboram dados publicados anteriormente, que apontam para um efeito adjuvante do DBP sobre as células dendríticas. Demonstramos maior indução de CD *in vitro*, aumento do recrutamento de neutrófilos *in vivo*, que poderia ser resultado de uma maior atividade apresentadora de antígenos, além de reportarmos uma reversão do efeito adjuvante do DBP após bloqueio dos receptores TRPA1 e TRPV1, expresso em células dendríticas.

Nossos dados contribuem para maior esclarecimento sobre os mecanismos moleculares envolvidos no efeito adjuvante do DBP.

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